INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/00, 9/64, A01K 67/027	**A1**	**(11) International Publication Number:** WO 97/20043 **(43) International Publication Date:** 5 June 1997 (05.06.97)

(21) International Application Number: PCT/US96/18866

(22) International Filing Date: 26 November 1996 (26.11.96)

(30) Priority Data:
08/565,074	30 November 1995 (30.11.95)	US
60/019,692	13 June 1996 (13.06.96)	US

(71) Applicants: ZYMOGENETICS, INC. [US/US]; 1201 Eastlake Avenue East, Seattle, WA 98102 (US). PPL THERAPEUTICS [GB/GB]; Roslin, Edinburgh, Midlothian EH25 9PP (GB).

(72) Inventors: GARNER, Ian; 13 Lismore Avenue, Edinburgh EHB 7DW (GB). COTTINGHAM, Ian; 9 Buckstone Row/Fairmilehead, Edinburgh EH10 6TW (GB). TEMPERLEY, Simon, M.; 12/4 Whitehorse Close, 27 Canon Gate, Edinburgh EH8 8BU (GB). FOSTER, Donald, C.; 3002 N.E. 181st Street, Seattle, WA 98155 (US). SPRECHER, Cindy, A.; 8207 39th Avenue N.E., Seattle, WA 98115 (US). PRUNKARD, Donna, E.; 1463 N.E. 92nd Street, Seattle, WA 98117 (US).

(74) Agent: SAWISLAK, Deborah, A.; ZymoGenetics, Inc., 1201 Eastlake Avenue East, Seattle, WA 98102 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: PROTEIN C PRODUCTION IN TRANSGENIC ANIMALS

(57) Abstract

Methods for producing protein C in transgenic non-human mammals are disclosed. The protein C is modified at the two-chain cleavage site between the light and heavy chains of protein C from Lys-Arg to R_1-R_2-R_3-R_4 where R_1 through R_4 are individually Arg or Lys. DNA segments encoding modified protein C are introduced into the germ line of a non-human mammal, and the mammal or its female progeny produces milk containing protein C expressed from the introduced DNA segments. The protein C expressed from the introduced DNA segments has anticoagulant activity when activated. Non-human mammalian embryos and transgenic non-human mammals carrying DNA segments encoding heterologous protein C are also disclosed.

DESCRIPTION
Protein C Production in Transgenic Animals

BACKGROUND OF THE INVENTION

    Protein C in its activated form plays an important role in regulating blood coagulation. The activated protein C, a serine protease, inactivates coagulation Factors Va and VIIIa by limited proteolysis. The coagulation cascade initiated by tissue injury, for example, is prevented from proceeding in an unimpeded chain-reaction beyond the area of injury by activated protein C.

    Protein C is synthesized in the liver as a single chain precursor polypeptide which is subsequently processed to a light chain of about 155 amino acids (M_r = 21,000) and a heavy chain of 262 amino acids (M_r = 40,000). The heavy and light chains circulate in the blood as a two-chain inactive protein, or zymogen, held together by a disulfide bond. When a 12 amino acid residue peptide is cleaved from the amino terminus of the heavy chain portion of the zymogen in a reaction mediated by thrombin, the protein becomes activated. The N-terminal portion of the light chain contains nine γ-carboxyglutamic acid (Gla) residues that are required for the calcium-dependent membrane binding and activation of the molecule. Another blood protein, referred to as "protein S", is believed to accelerate the protein C-catalyzed proteolysis of Factor Va.

    Protein C has also been implicated in the action of tissue-type plasminogen activator (Kisiel et al., Behring Inst. Mitt. 73:29-42, 1983). Infusion of bovine activated protein C (APC) into dogs results in increased plasminogen activator activity (Comp et al., J. Clin. Invest. 68:1221-1228, 1981). Other studies (Sakata et

al., _Proc. Natl. Acad. Sci. USA_ 82:1121-1125, 1985) have shown that addition of APC to cultured endothelial cells leads to a rapid, dose-dependent increase in fibrinolytic activity in the conditioned media, reflecting increases in the activity of both urokinase-related and tissue-type plasminogen activators. APC treatment also results in a dose-dependent decrease in anti-activator activity. In addition, studies with monoclonal antibodies against endogenous APC (Snow et al., FASEB Abstracts, 1988) implicate APC in maintaining patency of arteries during fibrinolysis and limiting the extent of tissue infarct.

Experimental evidence indicates that protein C may be clinically useful in the treatment of thrombosis. Several studies with baboon models of thrombosis have indicated that activated protein C in low doses will be effective in prevention of fibrin deposition, platelet deposition and loss of circulation (Gruber et al., _Hemostasis and Thrombosis_ 374a: abstract 1512, 1988; Widrow et al., _Fibrinolysis_ 2 suppl. 1: abstract 7, 1988; Griffin et al., _Thromb. Haemostasis_ 62: abstract 1512, 1989).

In addition, exogenous activated protein C has been shown to prevent the coagulopathic and lethal effects of gram negative septicemia (Taylor et al., _J. Clin. Invest._ 19:918-925, 1987). Data obtained from studies with baboons suggest that activated protein C plays a natural role in protecting against septicemia.

Until recently, protein C was purified from clotting factor concentrates (Marlar et al., _Blood_ 59:1067-1072, 1982) or from plasma (Kisiel, _J. Clin. Invest._ 64:761-769, 1979) and activated _in vitro_. However, the possibility that the resulting product could be contaminated with such infectious agents as hepatitis virus, cytomegalovirus, or human immunodeficiency virus (HIV) make the process unfavorable.

While expression of protein C through recombinant means has been theoretically possible as the

genes for both human and bovine protein C are known (Foster et al., _Proc. Natl. Acad. Sci. USA_ 82:4673-4677, 1985; Foster et al., _Proc. Natl. Acad Sci. USA_ 81:4766-4770, 1984 and U.S. Patent 4,775,624), it has been met

5 with limited success. Expression of some vitamin K-dependent proteins, such as protein C in cultured cells, has not produced protein C that has been at both commercially valuable levels and biologically functional when activated (i.e. had anticoagulant activity (Grinnell

10 et al., in Bruley and Drohn, eds., _Protein C and Related Anticoagulants_:29-63, Gulf Publishing, Houston, TX and Grinnell et al., _Bio/Technol._ 5:1189-1192, 1987)). Transgenic expression of protein C has yielded somewhat higher levels of expression, but the recombinant protein's

15 anticoagulant activity has still remained low, with less than 50% of the material having biological activity (Velander et al., _Proc. Natl. Acad. Sci. USA_ 89:12003-12007, 1992). Therefore, there remains a need for producing protein C that is both expressed at high levels

20 and has therapeutic value.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide methods for producing protein C in transgenic

25 animals. It is a further object to provide transgenic animals that express human protein C in a mammary gland.

Within one aspect, the present invention provides methods for producing protein C in a transgenic animal comprising (a) providing a DNA construct comprising

30 a first DNA segment encoding a secretion signal and a protein C propeptide operably linked to a second DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of R_1-R_4 is individually

35 Lys or Arg, and wherein said first and second segments are operably linked to additional DNA segments required for expression of the protein C DNA in a lactating mammary

gland of a host female animal; (b) introducing said DNA construct into a fertilized egg of a non-human mammalian species; (c) inserting said egg into an oviduct or uterus of a female of said species to obtain offspring carrying
5 said DNA construct; (d) breeding said offspring to produce female progeny that express said first and second DNA segments and produce milk containing protein C encoded by said second segment, wherein said protein has anticoagulant activity upon activation; (e) collecting
10 milk from said female progeny; and (f) recovering the protein C from the milk. In one embodiment, R_1-R_2-R_3-R_4 is Arg-Arg-Lys-Arg (SEQ ID NO: 20). In another embodiment, the method further comprises the step of activating the protein C. In another embodiment, the non-
15 human mammalian species is selected from sheep, rabbits, cattle and goats. In another embodiment each of the first and second DNA segments comprises an intron. In another embodiment, the second DNA segment comprises a DNA sequence of nucleotides as shown in SEQ ID NO: 1 or SEQ ID
20 NO:3. In another embodiment, the additional DNA segments comprise a transcriptional promoter selected from the group consisting of casein, β-lactoglobulin, α-lactoglobulin, α-lactalbumin and whey acidic protein gene promoters.

25   In another aspect, the present invention provides a transgenic non-human female mammal that produces recoverable amounts of human protein C in its milk, wherein at least 90% of the human protein C in the milk is two-chain protein C.

30   In another aspect, the present invention provides a process for producing a transgenic offspring of a mammal comprising the steps of (a) providing a DNA construct comprising a first DNA segment encoding a secretion signal and a protein C propeptide operably
35 linked to a second DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of

R_1-R_4 is individually Lys or Arg, and wherein said first and second segments are operably linked to additional DNA segments required for expression of the protein C DNA in a lactating mammary gland of a host female animal; (b)

5   introducing said DNA construct into a fertilized egg of a non-human mammalian species; and (c) inserting said egg into an oviduct or uterus of a female of said species to obtain offspring carrying said DNA construct.

Within another aspect, the present invention

10 provides non-human mammals produced according to the process for producing a transgenic offspring of a mammal comprising the steps of (a) providing a DNA construct comprising a first DNA segment encoding a secretion signal and a protein C propeptide operably linked to a second DNA

15 segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of R_1-R_4 is individually Lys or Arg, and wherein said first and second segments are operably linked to additional DNA segments required for

20 expression of the protein C DNA in a lactating mammary gland of a host female animal; (b) introducing said DNA construct into a fertilized egg of a non-human mammalian species; and (c) inserting said egg into an oviduct or uterus of a female of said species to obtain offspring

25 carrying said DNA construct.

In another aspect, the present invention provides a non-human mammalian embryo containing in its nucleus a heterologous DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain

30 cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of R_1-R_4 is individually Lys or Arg.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates analysis of plasma-derived

35 and transgenic protein C run under non-reducing and reducing conditions. Lane 1 is plasma-derived protein C

and lane 2 is transgenic protein C from the milk of sheep 30851.

Figure 2 illustrates sequencing of protein C from sheep line 30851. The initial yields were prosequence=9 pmol, light chain=563 pmol and heavy chain=565 pmol.

Figure 3 illustrates clotting activity of transgenic protein C compared to plasma-derived protein C.

DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention in detail, it will be helpful to define certain terms used herein:

As used herein, the term "biologically active" is used to denote protein C that is characterized by its anticoagulant and fibrinolytic properties. Protein C, when activated, inactivates factor Va and factor VIIIa in the presence of phospholipid and calcium. Activated protein C also enhances fibrinolysis, an effect believed to be mediated by the lowering of the levels of plasminogen activator inhibitors. As stated previously, two-chain protein C is activated upon cleavage of a 12 amino acid peptide from the amino terminus of the heavy chain portion of the zymogen.

The term "egg" is used to denote an unfertilized ovum, a fertilized ovum prior to fusion of the pronuclei or an early stage embryo (fertilized ovum with fused pronuclei).

A "female mammal that produces milk containing biologically active protein C" is one that, following pregnancy and delivery, produces, during the lactation period, milk containing recoverable amounts of protein C that can be activated to be biologically active. Those skilled in the art will recognized that such animals will naturally produce milk, and therefore the protein C, discontinuously.

The term "progeny" is used in its usual sense to include offspring and descendants.

The term "heterologous" is used to denote genetic material originating from a different species than that into which it has been introduced, or a protein produced from such genetic material.

Within the present invention, transgenic animal technology is employed to produce protein C within a mammary gland of a host female mammal. Expression in the mammary gland and subsequent secretion of the protein of interest into the milk overcomes many difficulties encountered in isolating proteins from other sources. Milk is readily collected, available in large quantities, and well characterized biochemically. Furthermore, the major milk proteins are present in milk at high concentrations (from about 1 to 16 g/l).

From a commercial point of view, it is clearly preferable to use as the host a species that has a large milk yield. While smaller animals such as mice and rats can be used (and are preferred at the proof-of-concept stage), within the present invention it is preferred to use livestock mammals including sheep and cattle. Sheep are particularly preferred due to such factors as the previous history of transgenesis in this species, milk yield, generation time, cost and the ready availability of equipment for collecting sheep milk. It is generally desirable to select a breed of host animal that has been bred for dairy use, such as East Friesland sheep, or to introduce dairy stock by breeding of the transgenic line at a later date. In any event, animals of known, good health status should be used.

Cloned DNA sequences encoding human protein C have been described (Foster and Davie, _Proc. Natl. Acad. Sci. USA_ 81:4766-4770, 1984; Foster et al., _Proc. Natl. Acad. USA_ 82:4673-4677, 1985; and Bang et al., U.S. Patent 4,755,624, each incorporated herein by reference). Complementary cDNAs encoding protein C can be obtained from libraries prepared from liver cells of various mammalian species according to standard laboratory

procedures. DNAs from other species, such as the protein C encoded by rats, pigs, sheep, cows and primates can be used and can be identified using probes from human cDNA.

In a preferred embodiment, human genomic DNAs encoding protein C are used. The human protein C gene is composed of nine exons ranging in size from 25 to 885 nucleotides, and seven introns ranging in size from 92 to 2668 nucleotides (U.S. Patent 4,959,318, incorporated herein by reference). The first exon is non-coding and referred to as exon O. Exon I and a portion of exon II code for the 42 amino acid signal sequence and propeptide (i.e., pre-propeptide). The remaining portion of exon II, exon III, exon IV, exon V and a portion of exon VI code for the light chain of protein C. The remaining portion of exon VI, exon VII and exon VIII code for the heavy chain of protein C. A representative human genomic DNA sequence and corresponding amino acid sequence are shown in SEQ ID NOS: 1 and 2, respectively. A representative human protein C cDNA sequence and corresponding amino acid sequences are shown in SEQ ID NO: 3 and 4, respectively.

Those skilled in the art will recognize that naturally occurring allelic variants of these sequences will exist; that additional variants can be generated by amino acid substitution, deletion, or insertion; and that such variants are useful within the present invention. In general, it is preferred that any engineered variants comprise only a limited number of amino acid substitutions, deletions, or insertions, and that any substitutions are conservative. Thus, it is preferred to produce protein C polypeptides that are at least 90%, and more preferably at least 95% or more identical in sequence to the corresponding native protein.

Within the present invention, the proteolytic processing involved in the maturation of recombinant protein C from single chain form to the two-chain form (i.e., cleaved between the light chain and the heavy chain) has been enhanced by modifying the amino acid

sequence around the two-chain cleavage site. In the
normal situation, endoproteolytic cleavage of the
precursor molecule at the Arg_{157}-Asp_{158} bond and the
removal of the dipeptide Lys_{156}-Arg_{157} by a
5　carboxypeptidase activity generate the light and heavy
chains of protein C prior to secretion. Expression of
protein C with the native (Lys-Arg) two-chain cleavage
site produces protein C that may contain up to 40% or more
uncleaved, single-chain protein C (Grinnel et al., in
10　Protein C and Related Anticoagulants, eds., Bruley and
Drohan, Gulf, Houston, pp. 29-63, 1990; Suttie, Thromb.
Res. 44:129-134, 1986 and Yan et al., Trends Biochem. Sci.
14:264-268, 1989). The single-chain form of protein C may
not be able to be activated. The cleavage site may be in
15　the form of the amino acid sequence R_1-R_2-R_3-R_4, wherein
each of R1 through R4 is individually lysine (Lys) or
arginine (Arg). Particularly preferred sequences include
Arg-Arg-Lys-Arg (SEQ ID NO: 20) and Lys-Arg-Lys-Arg (SEQ
ID NO: 21).

20　　　　　In a preferred embodiment, the present invention
provides for recoverable amounts of human protein C in the
milk of a non-human mammal, where at least 90%, preferably
at least 95%, of the human protein C is two-chain protein
C.

25　　　　　To obtain expression in the mammary gland, a
transcription promoter from a milk protein gene is used.
Milk protein genes include those genes encoding caseins,
beta-lactoglobulin (BLG), α-lactalbumin, and whey acidic
protein. The beta-lactoglobulin promoter is preferred.
30　In the case of the ovine beta-lactoglobulin gene, a region
of at least the proximal 406 bp of 5' flanking sequence of
the ovine BLG gene (contained within nucleotides 3844 to
4257 of SEQ ID NO: 5) will generally be used. Larger
portions of the 5' flanking sequence, up to about 5 kb,
35　are preferred. A larger DNA segment encompassing the 5'
flanking promoter region and the region encoding the 5'
non-coding portion of the beta-lactoglobulin gene

(contained within nucleotides 1 to 4257 of SEQ ID NO: 5) is particularly preferred. See Whitelaw et al., _Biochem J. 286_: 31-39, 1992. Similar fragments of promoter DNA from other species are also suitable.

5   Other regions of the beta-lactoglobulin gene may also be incorporated in constructs, as may genomic regions of the gene to be expressed. It is generally accepted in the art that constructs lacking introns, for example, express poorly in the transgenic lactating mammary gland

10 in comparison with those constructs that contain introns (see Brinster et al., _Proc. Natl. Acad. Sci. USA 85_: 836-840, 1988; Palmiter et al., _Proc. Natl. Acad. Sci. USA 88_: 478-482, 1991; Whitelaw et al., _Transgenic Res. 1_: 3-13, 1991; WO 89/01343; WO 91/02318). In this regard, it is

15 generally preferred, where possible, to use genomic sequences containing all or some of the native introns of a gene encoding protein C. Within certain embodiments of the invention, the further inclusion of at least some introns from the beta-lactoglobulin gene is preferred.

20 One such region is a DNA segment which provides for intron splicing and RNA polyadenylation from the 3' non-coding region of the ovine beta-lactoglobulin gene. When substituted for the natural 3' non-coding sequences of a gene, this ovine beta-lactoglobulin segment can both

25 enhance and stabilize expression levels of the protein C.

          For expression of protein C, DNA segments encoding protein C are operably linked to additional DNA segments required for their expression to produce expression units. One such additional segment is the

30 above-mentioned milk protein gene promoter. Sequences allowing for termination of transcription and polyadenylation of mRNA may also be incorporated. Such sequences are well known in the art, for example, one such termination sequence is the "upstream mouse sequence"

35 (McGeady et al., _DNA 5_:289-298,1986). The expression units will further include a DNA segment encoding a secretion signal operably linked to the segment encoding

the protein C polypeptide chain. The secretion signal may
be a native protein C secretion signal or may be that of
another protein, such as a milk protein. The term
"secretion signal" is used herein to denote that portion
of a protein that directs it through the secretory pathway
of a cell to the outside. Secretion signals are most
commonly found at the amino termini of proteins. See, for
example, von Heinje, Nuc. Acids Res. 14: 4683-4690, 1986;
and Meade et al., U.S. Patent No. 4,873,316, which are
incorporated herein by reference.

Construction of expression units is conveniently
carried out by inserting a protein C sequence into a
plasmid or phage vector containing the additional DNA
segments, although the expression unit may be constructed
by essentially any sequence of ligations. It is
particularly convenient to provide a vector containing a
DNA segment encoding a milk protein and to replace the
coding sequence for the milk protein with that of a
protein C (including a secretion signal), thereby creating
a gene fusion that includes the expression control
sequences of the milk protein gene. In any event, cloning
of the expression units in plasmids or other vectors
facilitates the amplification of the protein C sequences.
Amplification is conveniently carried out in bacterial
(e.g. E. coli) host cells, thus the vectors will typically
include an origin of replication and a selectable marker
functional in bacterial host cells.

The expression unit is then introduced into
fertilized eggs (including early-stage embryos) of the
chosen host species. Introduction of heterologous DNA can
be accomplished by one of several routes, including
pronuclear microinjection (e.g. U.S. Patent No.
4,873,191), retroviral infection (Jaenisch, Science 240:
1468-1474, 1988) or site-directed integration using
embryonic stem (ES) cells (reviewed by Bradley et al.,
Bio/Technology 10: 534-539, 1992). The eggs are then
implanted into the oviducts or uteri of pseudopregnant

females and allowed to develop to term. Offspring
carrying the introduced DNA in their germ line can pass
the DNA on to their progeny in the normal, Mendelian
fashion, allowing the development of transgenic herds.
5 General procedures for producing transgenic animals are
known in the art. See, for example, Hogan et al.,
Manipulating the Mouse Embryo: A Laboratory Manual, Cold
Spring Harbor Laboratory, 1986; Simons et al.,
Bio/Technology _6_: 179-183, 1988; Wall et al., _Biol._
10 _Reprod._ _32_: 645-651, 1985; Buhler et al., _Bio/Technology_
_8_: 140-143, 1990; Ebert et al., _Bio/Technology_ _9_: 835-838,
1991; Krimpenfort et al., _Bio/Technology_ _9_: 844-847, 1991;
Wall et al., _J. Cell. Biochem._ _49_: 113-120, 1992; and WIPO
publications WO 88/00239, WO 90/05188, WO 92/11757; and GB
15 87/00458, which are incorporated herein by reference.
Techniques for introducing foreign DNA sequences into
mammals and their germ cells were originally developed in
the mouse. See, e.g., Gordon et al., _Proc. Natl. Acad._
Sci. USA _77_: 7380-7384, 1980; Gordon and Ruddle, _Science_
20 _214_: 1244-1246, 1981; Palmiter and Brinster, _Cell_ _41_: 343-
345, 1985; Brinster et al., _Proc. Natl. Acad. Sci. USA_ _82_:
4438-4442, 1985; and Hogan et al. (ibid.). These
techniques were subsequently adapted for use with larger
animals, including livestock species (see e.g., WIPO
25 publications WO 88/00239, WO 90/05188, and WO 92/11757;
and Simons et al., _Bio/Technology_ _6_: 179-183, 1988). To
summarize, in the most efficient route used to date in the
generation of transgenic mice or livestock, several
hundred linear molecules of the DNA of interest are
30 injected into one of the pro-nuclei of a fertilized egg.
Injection of DNA into the cytoplasm of a zygote can also
be employed.

In general, female animals are superovulated by
treatment with follicle stimulating hormone, then mated.
35 Fertilized eggs are collected, and the heterologous DNA is
injected into the eggs using known methods. See, for
example, U.S. Patent No. 4,873,191; Gordon et al., _Proc._

Natl. Acad. Sci. USA 77: 7380-7384, 1980; Gordon and
Ruddle, Science 214: 1244-1246, 1981; Palmiter and
Brinster, Cell 41: 343-345, 1985; Brinster et al., Proc.
Natl. Acad. Sci. USA 82: 4438-4442, 1985; Hogan et al.,
Manipulating the Mouse Embryo: A Laboratory Manual, Cold
Spring Harbor Laboratory, 1986; Simons et al.
Bio/Technology 6: 179-183, 1988; Wall et al., Biol.
Reprod. 32: 645-651, 1985; Buhler et al., Bio/Technology
8: 140-143, 1990; Ebert et al., Bio/Technology 9: 835-838,
1991; Krimpenfort et al., Bio/Technology 9: 844-847, 1991;
Wall et al., J. Cell. Biochem. 49: 113-120, 1992; WIPO
publications WO 88/00239, WO 90/05118, and WO 92/11757;
and GB 87/00458, which are incorporated herein by
reference.

For injection into fertilized eggs, the
expression units are removed from their respective vectors
by digestion with appropriate restriction enzymes. For
convenience, it is preferred to design the vectors so that
the expression units are removed by cleavage with enzymes
that do not cut either within the expression units or
elsewhere in the vectors. The expression units are
recovered by conventional methods, such as electro-elution
followed by phenol extraction and ethanol precipitation,
sucrose density gradient centrifugation, or combinations
of these approaches.

DNA is injected into eggs essentially as
described in Hogan et al., ibid. In a typical injection,
eggs in a dish of an embryo culture medium are located
using a stereo zoom microscope (x50 or x63 magnification
preferred). Suitable media include Hepes (N-2-
hydroxyethylpiperazine-N'-2-ethanesulphonic acid) or
bicarbonate buffered media such as M2 or M16 (available
from Sigma Chemical Co., St. Louis, USA) or synthetic
oviduct medium (disclosed below). The eggs are secured
and transferred to the center of a glass slide on an
injection rig using, for example, a drummond pipette
complete with capillary tube. Viewing at lower (e.g. x4)

magnification is used at this stage. Using the holding pipette of the injection rig, the eggs are positioned centrally on the slide. Individual eggs are sequentially secured to the holding pipette for injection. For each

5 injection process, the holding pipette/egg is positioned in the center of the viewing field. The injection needle is then positioned directly below the egg. Preferably using x40 Nomarski objectives, both manipulator heights are adjusted to focus both the egg and the needle. The

10 pronuclei are located by rotating the egg and adjusting the holding pipette assembly as necessary. Once the pronucleus has been located, the height of the manipulator is altered to focus the pronuclear membrane. The injection needle is positioned below the egg such that the

15 needle tip is in a position below the center of the pronucleus. The position of the needle is then altered using the injection manipulator assembly to bring the needle and the pronucleus into the same focal plane. The needle is moved, via the joy stick on the injection

20 manipulator assembly, to a position to the right of the egg. With a short, continuous jabbing movement, the pronuclear membrane is pierced to leave the needle tip inside the pronucleus. Pressure is applied to the injection needle via, for example, a glass syringe until

25 the pronucleus swells to approximately twice its volume. At this point, the needle is slowly removed. Reverting to lower (e.g. x4) magnification, the injected egg is moved to a different area of the slide, and the process is repeated with another egg.

30   After the DNA is injected, the eggs may be cultured to allow the pronuclei to fuse, producing one-cell or later stage embryos. In general, the eggs are cultured at approximately the body temperature of the species used in a buffered medium containing balanced

35 salts and serum. Surviving embryos are then transferred to pseudopregnant recipient females, typically by inserting them into the oviduct or uterus, and allowed to

develop to term. During embryogenesis, some of the injected DNA integrates in a random fashion in the genomes of a small number of the developing embryos.

Potential transgenic offspring are screened via
5 blood samples and/or tissue biopsies. DNA is prepared from these samples and examined for the presence of the injected construct by techniques such as polymerase chain reaction (PCR; see Mullis, U.S. Patent No. 4,683,202) and Southern blotting (Southern, J. Mol. Biol. 98:503, 1975;
10 Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, 1982). Founder transgenic animals, or G0s, may be wholly transgenic, having transgenes in all of their cells, or mosaic, having transgenes in only a subset of cells (see, for example,
15 Wilkie et al., Develop. Biol. 118: 9-18, 1986). In the latter case, groups of germ cells may be wholly or partially transgenic. In the latter case, the number of transgenic progeny from a founder animal will be less than the expected 50% predicted from Mendelian principles.
20 Founder G0 animals are grown to sexual maturity and mated to obtain offspring, or G1s. The G1s are also examined for the presence of the transgene to demonstrate transmission from founder G0 animals. In the case of male G0s, these may be mated with several non-transgenic
25 females to generate many offspring. This increases the chances of observing transgene transmission. Female G0 founders may be mated naturally, artificially inseminated or superovulated to obtain many eggs which are transferred to surrogate mothers. The latter course gives the best
30 chance of observing transmission in animals having a limited number of young. The above-described breeding procedures are used to obtain animals that can pass the DNA on to subsequent generations of offspring in the normal, Mendelian fashion, allowing the development of,
35 for example, colonies (mice), flocks (sheep), or herds (pigs, goats and cattle) of transgenic animals.

The milk from lactating G0 and G1 females is examined for the expression of the heterologous protein using immunological techniques such as ELISA (see Harlow and Lane, _Antibodies, A Laboratory Manual_, Cold Spring

5 Harbor Laboratory, 1988) and Western blotting (Towbin et al., _Proc. Natl. Acad. Sci. USA_ 76: 4350-4354, 1979). For a variety of reasons known in the art, expression levels of the heterologous protein will be expected to differ between individuals.

10 A satisfactory family of animals should satisfy three criteria: they should be derived from the same founder G0 animal; they should exhibit stable transmission of the transgene; and they should exhibit acceptably stable expression levels from generation to generation and

15 from lactation to lactation of individual animals. These principles have been demonstrated and discussed (Carver et al., _Bio/Technology_ 11: 1263-1270, 1993). Animals from such a suitable family are referred to as a "line." Initially, male animals, G0 or G1, are used to derive a

20 flock or herd of producer animals by natural or artificial insemination. In this way, many female animals containing the same transgene integration event can be quickly generated from which a supply of milk can be obtained.

The protein C is recovered from milk using

25 standard practices such as skimming, precipitation, filtration and protein chromatography techniques.

Protein C produced according to the present invention can be activated by removal of the activation peptide from the amino terminus of the heavy chain.

30 Activation can be achieved using methods that are well known in the art, for example, using α-thrombin (Marlar et al., _Blood_ 59:1067-1072, 1982), trypsin (Marlar et al., 1982, ibid.), Russel's viper venom factor X activator (Kisiel, _J. Clin. Invest._ 64:761-769, 1979) or

35 commercially available Protac C (American Diagnostica, NY, NY).

The protein C molecules provided by the present invention and pharmaceutical compositions thereof are particularly useful for administration to humans to treat a variety of conditions involving intravascular
5   coagulation. For instance, although deep vein thrombosis and pulmonary embolism can be treated with conventional anticoagulants, the activated protein C described herein may be used to prevent the occurrence of thromboembolic complications in identified high risk patients, such as
10 those undergoing surgery or those with congestive heart failure. Since activated protein C is more selective than heparin, being active in the body generally when and where thrombin is generated and fibrin thrombi are formed, activated protein C will be more effective and less likely
15 to cause bleeding complications than heparin when used prophylactically for the prevention of deep vein thrombosis. The dose of activated protein C for prevention of deep vein thrombosis is in the range of about 100 µg to 100 mg/day, and administration should
20 begin at least about 6 hours prior to surgery and continue at least until the patient becomes ambulatory. In established deep vein thrombosis and/or pulmonary embolism, the dose of activated protein C ranges from about 100 µg to 100 mg as a loading dose followed by
25 maintenance doses ranging from 3 to 300 mg/day. Because of the lower likelihood of bleeding complications from activated protein C infusions, activated protein C can replace or lower the dose of heparin during or after surgery in conjunction with thrombectomies or
30 embolectomies.

The activated protein C compositions of the present invention will also have substantial utility in the prevention of cardiogenic emboli and in the treatment of thrombotic strokes. Because of its low potential for
35 causing bleeding complications and its selectivity, activated protein C can be given to stroke victims and may prevent the extension of the occluding arterial thrombus.

The amount of activated protein C administered will vary with each patient depending on the nature and severity of the stroke, but doses will generally be in the range of those suggested below.

5   Pharmaceutical compositions of activated protein C provided herein will be a useful treatment in acute myocardial infarction because of the ability of activated protein C to enhance *in vitro* fibrinolysis. Activated protein C can be given with tissue plasminogen activator

10 or streptokinase during the acute phases of the myocardial infarction. After the occluding coronary thrombus is dissolved, activated protein C can be given for subsequent days or weeks to prevent coronary reocculsion. In acute myocardial infarction, the patient is given a loading dose

15 of at least about 1-500 mg of activated protein C, followed by maintenance doses of 1-100 mg/day.

Activated protein C is useful in the treatment of disseminated intravascular coagulation (DIC). Patients with DIC characteristically have widespread

20 microcirculatory thrombi and often severe bleeding problems which result from consumption of essential clotting factors. Because of its selectivity, activated protein C will not aggravate the bleeding problems associated with DIC, as do conventional anticoagulants,

25 but will retard or inhibit the formation of additional microvascular fibrin deposits.

The invention is further illustrated by the following non-limiting examples.

30   EXAMPLES

Example I

A.   Vector pMAD6 Construction

The multiple cloning site of the vector pUC18

35 (Yanisch-Perron et al., Gene 33:103-119, 1985) was removed and replaced with a synthetic double stranded oligonucleotide (the strands of which are shown in SEQ ID

NO: 6 and SEQ ID NO: 7) containing the restriction sites Pvu I/Mlu I/Eco RV/Xba I/Pvu I/Mlu I, and flanked by 5' overhangs compatible with the restriction sites Eco RI and Hind III. pUC18 was cleaved with both Eco RI and Hind

5 III, the 5' terminal phosphate groups were removed with calf intestinal phosphatase, and the oligonucleotide was ligated into the vector backbone. The DNA sequence across the junction was confirmed by sequencing, and the new plasmid was called pUCPM.

10   The b-lactoglobulin (BLG) gene sequences from pSS1tgXS (disclosed in WIPO publication WO 88/00239) were excised as a Sal I-Xba I fragment and recloned into the vector pUCPM that had been cut with Sal I and Xba I to construct vector pUCXS. pUCXS is thus a pUC18 derivative

15 containing the entire BLG gene from the Sal I site to the Xba I site of phage SS1 (Ali and Clark, J. Mol. Biol. 199: 415-426, 1988).

        The plasmid pSS1tgSE (disclosed in WIPO publication WO 88/00239) contains a 1290 bp BLG fragment

20 flanked by Sph I and EcoR I restriction sites, a region spanning a unique Not I site and a single Pvu II site which lies in the 5' untranslated leader of the BLG mRNA. Into this Pvu II site was ligated a double stranded, 8 bp DNA linker (5'-GGATATCC-3') encoding the recognition site

25 for the enzyme Eco RV. This plasmid was called pSS1tgSE/RV. DNA sequences bounded by Sph I and Not I restriction sites in pSS1tgSE/RV were excised by enzymatic digestion and used to replace the equivalent fragment in pUCXS. The resulting plasmid was called pUCXSRV. The

30 sequence of the BLG insert in pUCXSRV is shown in SEQ ID NO: 5, with the unique Eco RV site at nucleotide 4245 in the 5' untranslated leader region of the BLG gene. This site allows insertion of any additional DNA sequences under the control of the BLG promoter 3' to the

35 transcription initiation site.

        Using the primers BLGAMP3 (5'-TGG ATC CCC TGC CGG TGC CTC TGG-3'; SEQ ID NO: 8) and BLGAMP4 (5'-AAC GCG

TCA TCC TCT GTG AGC CAG-3'; SEQ ID NO: 9) a PCR fragment of approximately 650 bp was produced from sequences immediately 3' to the stop codon of the BLG gene in pUCXSRV. The PCR fragment was engineered to have a BamH I

5 site at its 5' end and an Mlu I site at its 3' end and was cloned as such into BamH I and Mlu I cut pGEM7zf(+) (Promega) to give pDAM200(+).

pUCXSRV was digested with Kpn I, and the largest, vector containing band was gel purified. This

10 band contained the entire pUC plasmid sequences and some 3' non-coding sequences from the BLG gene. Into this backbone was ligated the small Kpn I fragment from pDAM200(+) which, in the correct orientation, effectively engineered a Bam HI site at the extreme 5' end of the 2.6

15 Kbp of the BLG 3' flanking region. This plasmid was called pBLAC200. A 2.6 Kbp Cla I-Xba I fragment from pBLAC200 was ligated into Cla I-Xba I cut pSP72 vector (Promega), thus placing an Eco RV site immediately upstream of the BLG sequences. This plasmid was called

20 pBLAC210.

The 2.6 Kbp Eco RV-Xba I fragment from pBLAC210 was ligated into Eco RV-Xba I cut pUCXSRV to form pMAD6 (SEQ ID NO: 23). This, in effect, excised all coding and intron sequences from pUCXSRV, forming a BLG minigene

25 consisting of 4.2 Kbp of 5' promoter and 2.6 Kbp of 3' downstream sequences flanking a unique Eco RV site. An oligonucleotide linker (ZC6839: ACTACGTAGT; SEQ ID NO: 10) was inserted into the Eco RV site of pMAD6 (SEQ ID NO: 23). This modification destroyed the Eco RV site and

30 created a Sna BI site to be used for cloning purposes. The vector was designated pMAD6-Sna. Messenger RNA initiates upstream of the Sna BI site and terminates downstream of the Sna BI site. The precursor transcript will encode a single BLG-derived intron, intron 6, which

35 is entirely within the 3' untranslated region of the gene.

B.   Intronless Vector pMAD

The beta-lactoglobulin cloning vector pMAD was also constructed to allow the insertion of cDNAs under the control of the beta-lactoglobulin gene promoter in
5   constructs containing no introns.   To generate pMAD, the plasmid pBLAC100 was opened by digestion with both Eco RV and Sal I.   The vector fragment was gel purified and the linearized vector was ligated with the 4.2 kb promoter fragment from the plasmid pUCXSRV as a Sal I-Eco RV
10 fragment.   The resulting construct was designated pST1 and constitutes a beta-lactoglobulin mini-gene encompassing a 4.2 kb of promoter region and 2.1 kb of 3' non-coding region beginning immediately downstream of the beta-lactoglobuling translational termination codon.   A unique
15 Eco RV site allows blunt-end cloning of any additional DNA sequences.   To generate transgenic animals it is generally accepted in the art and preferred to separate bacterial plasmid vector sequences from those intended to be used in the generation of transgenic animals.   In order to allow
20 the practical excision of novel cDNA based constructs using this beta-lactoglobulin mini-gene, the minigene was excised from pST1 on a Xho I-Not I fragment, the DNA termini made flush with Klenow polymerase and the product was ligated into the Eco RV site of pUCPM to yield pMAD.
25 Digestion with Mlu I liberates beta-lactoglobulin-cDNA constructs from the bacterial vector backbone.

Intronless constructs based on cDNAs and vectors such as pMAD benefit from the use of "rescue technology" for efficient expression.   Rescue technology takes
30 advantage of the ability of a co-injected and co-integrated BLG gene to improve the expression levels obtained from intronless, cDNA-based constructs in the transgenic system.   Rescue technology is disclosed in WIPO publication WO 92/11358, and is incorporated herein by
35 reference.

Example 2

A.   Isolation of cDNA

        A cDNA sequence coding for human protein C was
prepared as described in U.S. Patent 4,959,318, which is
5   incorporated herein by reference.   Briefly, a genomic
fragment containing an exon corresponding to amino acids -
42 to -19 (SEQ ID NO: 1) of the pre-pro peptide of protein
C was isolated, nick translated and used as a probe to
screen a cDNA library constructed by the technique of
10 Gubler and Hoffman, Gene 25:263-269, 1983, using mRNA from
HepG2 cells.   This cell line was derived from human
hepatocytes and was previously shown to synthesize protein
C (Fair and Bahnak, Blood 64:194-204, 1984).   Positive
clones comprising cDNA inserted into the Eco RI site of
15 phage λgt11 were isolated and screened with an
oligonucleotide probe corresponding to the 5' non-coding
region of the protein C gene.   One clone was also positive
with this probe and its entire nucleotide sequence was
determined.   The cDNA contained 70 bp of 5' untranslated
20 sequence, the entire coding sequence for human prepro-
protein C, and the entire 3' non-coding region
corresponding to the second polyadenylation site.

B.   Subcloning of Protein C cDNA

        The vector pDX was derived from pD3, which was
25 generated from plasmid pDHFRIII (Berkner et al., Nuc.
Acids Res. 13:841-857, 1985).   The Pst I site immediately
upstream from the DHFR sequence in pDHFRIII was converted
to a Bcl I site by digestion with Pst I.   The DNA was
phenol extracted, ethanol precipitated and resuspended in
30 buffer B (50 mM Tris pH 8, 7 mM MgCl$_2$, 7 mM β-MSH).   A
ligation reaction containing the linearized plasmid DNA
and Bcl I linkers was done.   The resulting plasmid was
phenol extracted, ethanol precipitated and digested with
Bcl I and gel purified.   The gel purified plasmid DNA was
35 circularized by ligation and used to transform E. coli
HB101.   Positive colonies were identified by restriction

analysis and designated pDHFR'. DNA from positive colonies was isolated and used to transform dam⁻ <u>E. coli</u>.

Plasmid pD2' was generated by cleaving pDHFR' and pSV40 (comprising Bam HI digested SV40 DNA cloned into

5 the Bam HI site of pML-1 (Lusky et al., <u>Nature</u> <u>293</u>:79-81, 1981)) with Bcl I and Bam HI. The DNA fragments were resolved by gel electrophoresis, and the 4.9 kb pDHFR' fragment and 0.2 kb SV40 fragment were isolated. These fragments were used in a ligation reaction, and the

10 resulting plasmid, designated pD2', was used to transform <u>E. coli</u> RRI.

Plasmid pD2' was modified by deleting the "poison" sequences in the pBR322 region (Lusky et al., 1981, ibid.). Plasmids pD2' and pML-1 were digested with

15 Eco RI and Nru I. The 1.7 kb pD2' fragment and 1.8 kb pML-1 fragment were isolated by gel purification, circularized in a ligation reaction and used to transform <u>E. coli</u> HB101. Positive colonies were identified using restriction analysis (designated pD2) and digested with

20 Eco RI and Bcl I. A 2.8 kb fragment (fragment C) was isolated and gel purified.

To generate the remaining fragments used in constructing pD3, pDHFRIII was modified to convert the Sac II (Sst II) site into either a Hind III or Kpn I site.

25 pDHFRIII was digested with Sst II and ligation reactions with either Hind III or Kpn I linkers were done. The resultant plasmids were digested with either Hind III or Kpn I and gel purified. The resultant plasmids were designated either pDHFRIII (Hind III) or pDHFRIII (Kpn I).

30 A 700 bp KpnI-Bgl II fragment (fragment A) was purified from pDHFRIII (Hind III).

The SV40 enhancer sequence was inserted into pDHFRIII (Hind III) by first digesting SV40 DNA with Hind III, and DNA from 5089 to 968 bp was isolated and

35 purified. Plasmid pDHFRIII (Hind III) was phosphatased, and the SV40 DNA and linearized plasmid pDHFRIII (Hind III) were used in a ligation reaction. A 700 bp Eco RI-

Kpn I fragment (fragment B) was isolated from the resulting plasmid.

For the final construction of pD3, fragments A (50 ng), B (50 ng) and C (10 ng) were combined in a ligation reaction and used to transform E. coli RRI. Positive colonies were isolated and plasmid DNA was prepared.

Plasmid pD3 was modified to accept the insertion of the protein C sequence by converting the Bcl I insertion site to an Eco RI site. First, the Eco RI site present in pD3 (the leftmost terminus in adenovirus 5 0-1) was converted to a Bam HI site via conventional linkering procedures. The resultant plasmid was transformed in E. coli HB101. Plasmid DNA was prepared, and positive clones were identified by restriction analysis.

pD3' is a vector identical to pD3 except that the SV40 polyadenylation signal (i.e., the SV40 Bam HI (2533 bp) to Bcl I (2770 bp) fragment) is in the late orientation. Thus, pD3' contains a Bam HI site as the site of gene insertion.

To generate pDX, the Eco RI site in pD3' was converted to a Bcl I site by Eco RI cleavage, incubation with SI nuclease and subsequent ligation with Bcl I linkers. DNA was prepared from a positively identified colony, and a 1.9 kb Xho I-Pst I fragment containing the altered restriction site was prepared via gel purification. In a second modification, Bcl I-cleaved pD3 was ligated with Eco RI-Bcl I adapters in order to generate an Eco RI site as the position for inserting a gene into the expression vector. Positive colonies were identified by restriction analysis. The resulting plasmid, designated pDX, has a unique Eco RI site for insertion of foreign genes.

The protein C cDNA was inserted into pDX as an Eco RI fragment. Plasmids were screened by restriction analysis. A plasmid having the protein C insert in the correct orientation with respect to the promoter elements

and plasmid DNA was designated pDX/PC. Because the cDNA insert in pDX/PC contains a ATG codon in the 5' non-coding region, deletion mutagenesis was performed on the cDNA. Deletion of the three base pairs was performed according
5   to standard procedures or oligonucleotide-directed mutagenesis. The pDX-based vector containing the modified cDNA was designated p594.

C.   Modification of the Protein C Processing Site

10   To enhance the processing of single-chain protein C to the two-chain form, two additional arginine residues were introduced immediately upstream of the Lys$_{156}$-Arg$_{157}$ cleavage site of the precursor protein, resulting in a cleavage site consisting of four basic
15   amino acids, Arg-Arg-Lys-Arg (SEQ ID NO: 20). The resultant mutant precursor of protein C was designated PC962. It contains the sequence Ser-His-Leu-Arg-Arg-Lys-Arg-Asp (SEQ ID NO: 22) at the cleavage site. Processing at the Arg-Asp bond results in a two-chain protein C
20   molecule.

The mutant molecule was generated by altering the cloned cDNA by site-specific mutagenesis (essentially as described by Zoller and Smith, DNA 3:479-488, 1984, for the two-primer method) using the mutagenic oligonucleotide
25   ZC962 (5'AGTCACCTGAGAAGAAAACGAGACA3'; SEQ ID NO: 11). Plasmid p594 was digested with Sst I, and the approximately 87 bp fragment was cloned into M13mp11 and single-stranded template DNA was isolated. Following mutagenesis, a correct clone was identified by sequencing.
30   Replicative form DNA was isolated, digested with Sst I, and the protein C fragment was inserted into Sst I-cut p594. Clones having the Sst I fragment inserted in the desired orientation were identified by restriction enzyme mapping. The resulting expression vector was designated
35   pDX/PC962.

●

●

D.   <u>Intronless Protein C Construct</u>

To facilitate the cloning of the protein C cDNA, PC962, into pMAD, the cDNA contained in pCX/PC962 was modified to incorporate Eco RV sites at the extremities of the protein C cDNA insert. A 769 bp Sst II-Pst I fragment encompassing the 3' end of PC962 was cloned between the Sst II and Pst I sites of pBluescript II SK® (Stratagene, La Jolla, CA). The fragment was excised with Sst II and Eco RV and purified. The 5' portion of PC962 was modified by PCR. The sense oligonucleotide primer for this reaction covered the 5' ATG region of the cDNA and provided an Eco RV site upstream of this in the product. The antisense oligonucleotide primer covered the Sst II site used to generate the Sst II-Eco RV fragment. The resulting PCR product was digested with Eco RV and Sst II and ligated with the Sst II-Eco RV 3' fragment and Eco RV digested pMAD. The resulting plasmid, designated pCORP9 effectively contained the PC962 cDNA flanked by Eco RV sites in an intronless fusion driven by the beta-lactoglobulin promoter.

E.   <u>Genomic Protein C DNA Construction</u>

A genomic DNA construct containing exons I through VIII was made. See, U.S. Patent 4,959,318, which is incorporated herein by reference, for disclosure of the exon structure of the protein C gene. This genomic construct, designated GPC10-1, changed the sequence 16 base pairs upstream of the ATG from the native protein C sequence to the beta-lactoglobulin sequence and introduced mutations in the propeptide cleavage site located in exon 2, and the two-chain cleavage site located in exon 6, as described below.

The construct was assembled using four fragments designated A, B, C and D and encompassed the protein C gene sequence from the ATG to a Bam HI site in exon VIII, immediately upstream of the stop codon. The fragments were generated from a human genomic library in λ Charon 4A phage that was screened with a radiolabeled cDNA probe for

human protein C. The screening of the λ library produced three clones that together mapped the entire protein C gene (Foster et al., 1985, ibid.). These clones were designated PCλ1, PCλ6 and PCλ8.

5 Fragment A was a Not I to Eco RI fragment that contained exons I and II of the genomic sequence and was 1698 bp. A subclone of PCλ6 contained an Eco RI to Eco RI fragment and was designated pHCR4.4-1. Using pHCR4.4-1 as a template and oligonucleotides ZC6303 (SEQ ID NO: 12) and
10 ZC6337 (SEQ ID NO: 13), a DNA fragment was generated by polymerase chain reaction (PCR). Oligonucleotide ZC6303 changed the sequence 16 base pairs 5' to the ATG sequence from the native protein C sequence to the equivalent sequence from the beta-lactoglobulin gene and introduced a
15 Not I site. Oligonucleotide ZC6337 changed the propeptide cleavage site from Arg-Ile-Arg-Lys-Arg (SEQ ID NO: 24) to Gln-Arg-Arg-Lys-Arg (SEQ ID NO: 25). The resulting PCR-generated fragment was digested with Not I and Bss HII, and a 1402 base pair fragment was gel purified and
20 designated A1. A second fragment was prepared using a λ gt11 clone of PCλ1 as a template with oligonucleotides ZC6306 (SEQ ID NO: 14) and ZC6338 (SEQ ID NO: 15) in a polymerase chain reaction. The resulting DNA fragment, designated A3, was digested with Bss HII and Eco RI and
25 gel purified, resulting in a 296 base pair fragment. Fragments A1 and A3 were ligated into the Bluescript II KS ® phagemid vector (Stratagene, La Jolla, CA). The resulting plasmid, designated GPC 2-2, was digested with Not I and Eco RI, gel purified and the Not I-Eco RI DNA
30 fragment was designated Fragment A.

pCR 2-14 is a subclone that contains an Eco RI to Eco RI DNA fragment of PCλ8 (Foster et al., 1985, ibid.). The plasmid was digested with Eco RI and Sst I and gel purified. The resulting fragment was designated
35 Fragment B.

Plasmid pCR 2-14 was used as template DNA with oligonucleotides ZC6373 (SEQ ID NO: 16) and ZC6305 (SEQ ID

NO: 17), which introduced an Afl II site and the RRKR mutation of the native (KR) two-chain cleavage site, in a polymerase chain reaction. The resulting PCR-generated fragment was digested with Bgl II and Afl II and gel

5 purified, resulting in a 1441 base pair fragment, designated E1. Fragment E1 was used in a ligation reaction with oligonucleotides ZC6302 (SEQ ID NO: 18) and ZC6304 (SEQ ID NO: 19). These oligonucleotides form Afl II and Sst II restriction sites when annealed and were

10 ligated to the 3' end of fragment E1, resulting in a fragment with a 5' Bgl II site and a 3' Sst II site. This fragment was used in a ligation reaction with a Bam HI-Sst II digested Bluescript II KS® phagemid vector (Stratagene). The resulting plasmid was designated GPC 8-

15 5 and digested with Sst I and Sst II, generating a 626 base pair fragment, designated Fragment C.

A fourth fragment was generated by digestion of a genomic subclone (pHCB7-1) of PCλ8. pHCB7-1 contained a Bgl II to Bgl II fragment that encompassed exons VI

20 through VIII. pHCB7-1 was digested with Sst I and Bam HI and a 2702 base pair fragment was gel purified. The fragment was designated Fragment D.

A five-part ligation reaction was prepared using Not I and Bam HI digested and linearized Bluescript II KS®

25 phagemid vector (Stratagene) with Fragment A (5' Not I to 3' Eco RI) that contained exons I and II, Fragment B (5' Eco RI to 3' Sst I) that contained exons III. IV and V, Fragment C (5' Sst I to 3' Sst II) that contained the 5' portion of exon VI and Fragment D (5' Sst II to 3' Bam HI)

30 that contained the remaining 3' portion of exon VI and exons VII and VIII. The resulting DNA was 8950 base pairs and designated GPC 10-1.

GPC10-1 was originally generated with BLG sequences and a Not I site upstream of the ATG initiator

35 codon and modifications to both cleavage sites. A clone, designated pPC12/BS, was generated to ensure that the 5' Not I site of GPC10-1 would not introduce secondary

structure into mRNA molecules that could hinder translation. pPC12/BS was generated using PCR amplification of a 1 kb Not I-Sca I fragment that covered the 5' region of the protein C gene and contained the

5   wild-type ATG codon environment. This introduced an Eco RV site immediately downstream of the Not I site, adjacent to the ATG codon, and a Bam HI site was incorporated 3' of the Sca I site to facilitate cloning. Following a Not I/Bam HI digestion, the PCR product was cloned into Not I-

10   Bam HI digested Bluescript II KS® phagemid vector (Stratagene). The Not I-Eco RV-Sca I fragment present in pPC12/BS was excised, purified and ligated to GPC10-1, which had been linearized with Not I and partially digested with Sca I (the pUC ampillicin gene has an

15   internal Sca I site). The resulting clone was designated GPC10-2 and possesses an Eco RV site immediately upstream of the ATG initiator codon.

GPC10-1 and GPC10-2 both terminated at the final Bam HI site in exon VIII of the protein C gene. To

20   reconstitute the 56 bp of sequence, ending at the termination codon, two oligonucleotides were synthesized with flanking Bam HI (5') and Bgl II (3') restriction sites. Following annealing of the oligonucleotides, the product was cloned into Bam HI digested pBST+ to generate

25   plasmid pPC3'. pBST+ is a derivative of pBS (Stratagene) with a new polylinker. The addition of the polylinker added Bgl II, Xho I, Nar I and Cla I restriction sites from the vector polylinker downstream of the destroyed Bgl II site of the oligonucleotide construct.

30   The Not I-Bam HI fragment of GPC10-1 was subcloned into Not I/Bam HI digested pPC3' to add 3' coding sequences of protein C, the TAG termination codon followed by Bgl II-Xho I-Nar I-Cla I. The 3' region of the protein C gene beginning with the Eco RV site in

35   intron V was excised from this plasmid on an Eco RV-Cla I fragment.

The Eco RV-Eco RV fragment from GPC10-2, covering the 5' portion of the protein C gene, and the above Eco RI-Cla I fragment covering the 3' portion of the protein C gene were combined between the Eco RV and Cla I
5 sites of pMAD6 (SEQ ID NO: 23) to generate pCORP13. This effectively placed a genomic portion of the protein C gene with modified propeptide and two-chain cleavage sites under the control of the beta-lactoglobulin promoter.

A further genomic construct was generated from
10 pCORP13 that contained only the modified two-chain cleavage site. This was achieved using PCR amplification to modify two fragments which resulting in restoration of the coding capability of exon 2 from the mutant Gln-Arg-Arg-Lys-Arg (SEQ ID NO: 25) to the wild-type Arg-Ile-Arg-
15 Lys-Arg (SEQ ID NO: 24). pCORP13 was used as template for these reactions. The first fragment was 1.3 kb, which encompassed the 5' end of the protein C gene up to the Bam HI site in exon 2. For this reason, the sense primer was designed to add a Hind III site 5' to the Eco RV site
20 proximal to the ATG initiation codon. The antisense primer was designed to restore the wild-type sequences in exon 2, which included a restored Bam HI site. A second fragment of 0.2 kb from the Bam HI site in exon 2 to the Xho I site in intron 2, was also amplified. The two
25 fragments were combined in pGEMII (Promega, Madison, WI) to generate pGEMPC1.5. A 7.5 kb Xho I fragment from pCORP 13 was ligated to Xho I digested pGEMPC1.5 to generate a complete protein C genomic sequence covering exons 1-8 with a wild-type propeptide cleavage site and a modified
30 two-chain cleavage site. The plasmid was designated pGEMPC14. The sequence was excised from pGEMPC14 as a Hind III/Sal I fragment. The DNA termini were repaired using a Klenow reaction and the fragment was blunt-end ligated into Eco RV digested pMAD6 (SEQ ID NO: 23) to
35 generate pCORP14.

Example 3

Mice for initial breeding stocks (C57BL6J, CBACA) were obtained from Harlan Olac Ltd. (Bicester, UK). These were mated in pairs to produce F1 hybrid cross
5 (B6CBAF1) for recipient females, superovulated females, stud males and vasectomized males. All animals were kept on a 14 hour light/10 hour dark cycle and fed water and food (Special Diet Services RM3, Edinburgh, Scotland) *ad libitum.*

10 Transgenic mice were generated essentially as described in Hogan et al., Manipulating the Mouse Embryo: A Laboratory Manual, Cold Spring Harbor Laboratory, 1986, which is incorporated herein by reference in its entirety. Female B6CBAF1 animals were superovulated at 4-5 weeks of
15 age by an i.p. injection of pregnant mares' serum gonadotrophin (FOLLIGON, Vet-Drug, Falkirk, Scotland) (5 iu) followed by an i.p. injection of human chorionic gonadotrophin (CHORULON, Vet-Drug, Falkirk, Scotland) (5 iu) 45 hours later. They were then mated with a stud male
20 overnight. Such females were next examined for copulation plugs. Those that had mated were sacrificed, and their eggs were collected for microinjection.

DNA was injected into the fertilized eggs as described in Hogan et al. (ibid.). Briefly, the vector
25 containing the protein C expression unit was digested with Mlu I, and the expression unit was isolated by sucrose gradient centrifugation. All chemicals used were reagent grade (Sigma Chemical Co., St. Louis, MO, U.S.A.), and all solutions were sterile and nuclease-free. Solutions of
30 20% and 40% sucrose in 1 M NaCl, 20 mM Tris pH 8.0, 5 mM EDTA were prepared using UHP water and filter sterilized. A 30% sucrose solution was prepared by mixing equal volumes of the 20% and 40% solutions. A gradient was prepared by layering 0.5 ml steps of the 40%, 30% and 20%
35 sucrose solutions into a 2 ml polyallomer tube and allowed to stand for one hour. 100 µl of DNA solution (max. 8 µg DNA) was loaded onto the top of the gradient, and the

gradient was centrifuged for 17-20 hours at 26,000 rpm, 15°C in a Beckman TL100 ultracentrifuge using a TLS-55 rotor (Beckman Instruments, Fullerton, CA, USA). Gradients were fractionated by puncturing the tube bottom with a 20 ga. needle and collecting drops in a 96 well microtiter plate. 3 μl aliquots were analyzed on a 1% agarose mini-gel. Fractions containing the protein C DNA fragment were pooled and ethanol precipitated overnight at -20°C in 0.3M sodium acetate. DNA pellets were resuspended in 50-100 μl UHP water and quantitated by fluorimetry. The protein C expression unit was diluted in Dulbecco's phosphate buffered saline without calcium and magnesium (containing, per liter, 0.2 g KCl, 0.2 g KH_2PO_4, 8.0 g NaCl, 1.15 g Na_2HPO_4) or in TE (10 mM Tris-HCl, 1 mM EDTA pH 7.5). DNA concentration is adjusted to about 6 μg/ml, prior to injection into the eggs (~2 pl total DNA solution per egg).

Recipient females of 6-8 weeks of age are prepared by mating B6CBAF1 females in natural estrus with vasectomized males. Females possessing copulation plugs are then kept for transfer of microinjected eggs.

Following birth of potential transgenic animals, tail biopsies are taken, under anesthesia, at four weeks of age. Tissue samples are placed in 2 ml of tail buffer (0.3 M Na acetate, 50 mM NaCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl, pH 8.5, 0.5% NP40, 0.5% Tween 20) containing 200 μg/ml proteinase K (Boehringer Mannheim, Mannheim, Germany) and vortexed. The samples are shaken (250 rpm) at 55°-60°C for 3 hours to overnight. DNA prepared from biopsy samples is examined for the presence of the injected constructs by PCR and Southern blotting. The digested tissue is vigorously vortexed, and 5 μl aliquots are placed in 0.5 ml microcentrifuge tubes. Positive and negative tail samples are included as controls. Forty μl of silicone oil (BDH, Poole, UK) is added to each tube, and the tubes are briefly centrifuged. The tubes are incubated in the heating block of a thermal cycler (e.g.

Omni-gene, Hybaid, Teddington, UK) to 95°C for 10 minutes. Following this, each tube has a 45 ll aliquot of PCR mix added such that the final composition of each reaction mix is: 50 mM KCl; 2 mM $MgCl_2$; 10 mM Tris-HCl (pH 8.3); 0.01%

5   gelatin; 0.1% NP40, 10% DMSO; 500 nM each primer, 200 lM dNTPs; 0.02 U/ll Taq polymerase (Boehringer Mannheim, Mannheim, Germany). The tubes are then cycled through 30 repeated temperature changes as required by the particular primers used. The primers may be varied but in all cases

10 must target the BLG promoter region. This is specific for the injected DNA fragments because the mouse does not have a BLG gene. Twelve ll of 5x loading buffer containing Orange G marker dye (0.25% Orange G (Sigma) 15% Ficoll type 400 (Pharmacia Biosystems Ltd., Milton Keynes, UK))

15 is then added to each tube, and the reaction mixtures are electrophoresed on a 1.6% agarose gel containing ethidium bromide (Sigma) until the marker dye has migrated 2/3 of the length of the gel. The gel is visualized with a UV light source emitting a wavelength of 254 nm. Transgenic

20 mice having one or more of the injected DNA fragments are identified by this approach.

Positive tail samples are processed to obtain pure DNA. The DNA samples are screened by Southern blotting using a BLG promoter probe (nucleotides 2523-4253

25 of SEQ ID NO: 7).

Southern blot analysis of transgenic mice prepared essentially as described above demonstrated that approximately 10% of progeny contained protein C sequences. Examination of milk from positive animals by

30 reducing SDS polyacrylamide gel electrophoresis demonstrated the presence of protein C at concentrations up to 1 mg/ml.

Example 4

35 Donor ewes are treated with an intravaginal progesterone-impregnated sponge (CHRONOGEST Goat Sponge,

34

Intervet, Cambridge, UK) on day 0. Sponges are left *in situ* for ten or twelve days.

Superovulation is induced by treatment of donor
ewes with a total of one unit of ovine follicle
5 stimulating hormone (OFSH) (OVAGEN, Horizon Animal
Reproduction Technology Pty. Ltd., New Zealand)
administered in eight intramuscular injections of 0.125
units per injection starting at 5:00 pm on day -4 and
ending at 8:00 am on day 0. Donors are injected
10 intramuscularly with 0.5 ml of a luteolytic agent
(ESTRUMATE, Vet-Drug) on day -4 to cause regression of the
corpus luteum, to allow return to estrus and ovulation.
To synchronize ovulation, the donor animals are injected
intramuscularly with 2 ml of a synthetic releasing hormone
15 analog (RECEPTAL, Vet-Drug) at 5:00 pm on day 0.

Donors are starved of food and water for at
least 12 hours before artificial insemination (A.I.). The
animals are artificially inseminated by intrauterine
laparoscopy under sedation and local anesthesia on day 1.
20 Either xylazine (ROMPUN, Vet-Drug) at a dose rate of 0.05-
0.1 ml per 10 kg bodyweight or ACP injection 10 mg/ml
(Vet-Drug) at a dose rate of 0.1 ml per 10 kg bodyweight
is injected intramuscularly approximately fifteen minutes
before A.I. to provide sedation. A.I. is carried out
25 using freshly collected semen from a Poll Dorset ram.
Semen is diluted with equal parts of filtered phosphate
buffered saline, and 0.2 ml of the diluted semen is
injected per uterine horn. Immediately pre- or post-A.I.,
donors are given an intramuscular injection of AMOXYPEN
30 (Vet-Drug).

Fertilized eggs are recovered on day 2 following
starvation of donors of food and water from 5:00 pm on day
1. Recovery is carried out under general anesthesia
induced by an intravenous injection of 5% thiopentone
35 sodium (INTRAVAL SODIUM, Vet-Drug) at a dose rate of 3 ml
per 10 kg bodyweight. Anesthesia is maintained by
inhalation of 1-2% Halothane/O_2/N_2O. To recover the

fertilized eggs, a laparotomy incision is made, and the uterus is exteriorized. The eggs are recovered by retrograde flushing of the oviducts with Ovum Culture Medium (Advanced Protein Products, Brierly Hill, West

5 Midlands, UK) supplemented with bovine serum albumin of New Zealand origin. After flushing, the uterus is returned to the abdomen, and the incision is closed. Donors are allowed to recover post-operatively or are euthanized. Donors that are allowed to recover are given

10 an intramuscular injection of Amoxypen L.A. at the manufacturer's recommended dose rate immediately pre- or post-operatively.

Plasmids containing the protein C DNA are digested with Mlu I, and the expression unit fragments are

15 recovered and purified on sucrose density gradients. The fragment concentrations are determined by fluorimetry and diluted in Dulbecco's phosphate buffered saline without calcium and magnesium or TE as described above. The concentration is adjusted to 6 lg/ml, and approximately 2

20 pl of the mixture is microinjected into one pronucleus of each fertilized eggs with visible pronuclei.

All fertilized eggs surviving pronuclear microinjection are cultured in vitro at 38.5°C in an atmosphere of 5% CO_2:5% O_2:90% N_2 and about ~100% humidity

25 in a bicarbonate buffered synthetic oviduct medium (see Table) supplemented with 20% v/v vasectomized ram serum. The serum may be heat inactivated at 56°C for 30 minutes and stored frozen at -20°C prior to use. The fertilized eggs are cultured for a suitable period of time to allow

30 early embryo mortality (caused by the manipulation techniques) to occur. These dead or arrested embryos are discarded. Embryos having developed to 5 or 6 cell divisions are transferred to synchronized recipient ewes.

Table
Synthetic Oviduct Medium

Stock A (Lasts 3 Months)

NaCl	6.29 g
KCl	0.534 g
KH_2PO_4	0.162 g
$MgSO_4.7H_2O$	0.182 g
Penicillin	0.06 g
Sodium Lactate 60% syrup	0.6 mls
Super H_2O	99.4 mls

Stock B (Lasts 2 weeks)

$NaHCO_3$	0.21 g
Phenol red	0.001 g
Super H_2O	10 mls

Stock C (Lasts 2 weeks)

Sodium Pyruvate	0.051 g
Super H_2O	10 mls

Stock D (Lasts 3 months)

CaCl2.2H_2O	0.262 g
Super H_2O	10 mls

Stock E (Lasts 3 months)

Hepes	0.651 g
Phenol red	0.001 g
Super H_2O	10 mls

To make up 10mls of Bicarbonate Buffered Medium

STOCK A	1 ml
STOCK B	1 ml
STOCK C	0.07 ml
STOCK D	0.1 ml
Super H_2O	7.83 ml

Osmolarity should be 265-285 mOsm.
Add 2.5 ml of heat inactivated sheep serum
and filter sterilize.

To make up 10 mls of HEPES Buffered Medium

STOCK A	1 ml
STOCK B	0.2 ml
STOCK C	0.07 ml
STOCK D	0.1 ml
STOCK E	0.8 ml
Super H2O	7.83 ml

Table, cont.

Osmolarity should be 265-285 mOsm.
Add 2.5 ml of heat inactivated sheep serum
and filter sterilize.

Recipient ewes are treated with an intravaginal progesterone-impregnated sponge (Chronogest Ewe Sponge or Chronogest Ewe-Lamb Sponge, Intervet) left in situ for 10 or 12 days. The ewes are injected intramuscularly with 1.5 ml (300 iu) of a follicle stimulating hormone substitute (P.M.S.G., Intervet) and with 0.5 ml of a luteolytic agent (Estrumate, Coopers Pitman-Moore) at sponge removal on day -1. The ewes are tested for estrus with a vasectomized ram between 8:00 am and 5:00 pm on days 0 and 1.

Embryos surviving in vitro culture are returned to recipients (starved from 5:00 pm on day 5 or 6) on day 6 or 7. Embryo transfer is carried out under general anesthesia as described above. The uterus is exteriorized via a laparotomy incision with or without laparoscopy. Embryos are returned to one or both uterine horns only in ewes with at least one suitable corpora lutea. After replacement of the uterus, the abdomen is closed, and the recipients are allowed to recover. The animals are given an intramuscular injection of Amoxypen L.A. at the manufacturer's recommended dose rate immediately pre- or post-operatively.

Lambs are identified by ear tags and left with their dams for rearing. Ewes and lambs are either housed and fed complete diet concentrates and other supplements and or ad lib. hay, or are let out to grass.

Within the first week of life (or as soon thereafter as possible without prejudicing health), each lamb is tested for the presence of the heterologous DNA by two sampling procedures. Following tail biopsy, within a week, a 10 ml blood sample is taken from the jugular vein into an EDTA vacutainer. Tissue samples are taken by tail

biopsy as soon as possible after the tail has become desensitized after the application of a rubber elastrator ring to its proximal third (usually within 200 minutes after "tailing"). The tissue is placed immediately in a

5 solution of tail buffer. Tail samples are kept at room temperature and analyzed on the day of collection. All lambs are given an intramuscular injection of Amoxypen L.A. at the manufacturer's recommended dose rate immediately post-biopsy, and the cut end of the tail is

10 sprayed with an antibiotic spray.

DNA is extracted from sheep blood by first separating white blood cells. A 10 ml sample of blood is diluted in 20 ml of Hank's buffered saline (HES; obtained from Sigma Chemical Co.). Ten ml of the diluted blood is

15 layered over 5 ml of Histopaque (Sigma) in each of two 15 ml screw-capped tubes. The tubes are centrifuged at 3000 rpm (2000 x g max.), low brake for 15 minutes at room temperature. White cell interfaces are removed to a clean 15 ml tube and diluted to 15 ml in HBS. The diluted cells

20 are spun at 3000 rpm for 10 minutes at room temperature, and the cell pellet is recovered and resuspended in 2-5 ml of tail buffer.

To extract DNA from the white cells, 10% SDS is added to the resuspended cells to a final concentration of

25 1%, and the tube is inverted to mix the solution. One mg of fresh proteinase K solution is added, and the mixture is incubated overnight at 45°C. DNA is extracted using an equal volume of phenol/chloroform (x3) and chloroform/isoamyl alcohol (x1). The DNA is then

30 precipitated by adding 0.1 volume of 3 M NaOAc and 2 volumes of ethanol, and the tube is inverted to mix. The precipitated DNA is spooled out using a clean glass rod with a sealed end. The spool is washed in 70% ethanol, and the DNA is allowed to partially dry, then is

35 redissolved in TE (10 mM Tris-HCl, 1 mM EDTA, pH 7.5).

DNA samples from blood and tail are analyzed by Southern blotting using probes for the BLG promoter region and the protein C coding regions.

5 Example 5

A founder female animal, designated 30851, which is transgenic for both BLG and pCORP9 was generated. She has given rise to two sons and a transgenic daughter, designated 40387. Recombinant transgenic protein C was
10 purified from milk (from 30851) by a single chromatography step using a calcium-dependent monoclonal antibody affinity column. Briefly, the milk samples were pooled up to a volume of 40 ml. Two volumes of ice-cold 1 X TBS (50 mM Tris-HCl, 150 mM NaCl pH 6.5) and 200 mM EDTA, pH 6.5
15 were added to solubilise the caseins. The EDTA-treated milk solution was centrifuged at 15,000 rpm for 30 minutes at 4°C in a JA20 rotor (Beckman Instruments, Irvine, CA). After centrifugation, the upper lipid phase and the small pellet were discarded.
20 The EDTA-treated milk was diluted with an equal volume of ice-cold 1 X TBS and 133 mM $CaCl_2$ while stirring. A cloudy precipitate formed upon addition of the $CaCl_2$. The pH was quickly adjusted by addition of a few drops of 4 M NaOH, and the precipitate was
25 redissolved. Any remaining insoluble material was removed by filtration through a 0.45 μm filter.

The optical density of the solubilised milk was measured at 280 nm, and the protein concentration was calculated. The milk was diluted to a protein
30 concentration of 10 mg/ml using 1 X TBS containing $CaCl_2$ to give a final Ca^{++} concentration of 25 mM. The milk was used to resuspend antibody-Sepharose that carried the immobilized Ca^{++}-dependent monoclonal antibody PCL-2, and had been washed in 1 X TBS and 25 mM $CaCl_2$. PCL-2 is a
35 monoclonal antibody that binds single chain and two chain protein C, whether or not they are gamma-carboxylated. The milk-Sepharose mixture was incubated overnight at 4°C.

The matrix was washed twice in batch with 1 x TBS and 25 mM $CaCl_2$ and packed into a glass column. The resin was washed at a flow rate of 1 ml/min with a calcium containing buffer and a stable baseline was achieved
5 before the bound protein was eluted with an isocratic elution using 1 X TBS and 25 mM EDTA, pH 6.5. Fractions containing protein C were pooled and concentrated to approximately 1 ml using an Amicon ultrafiltration unit with a 10 kDa cut-off membrane (Amicon, Danvers, MA).
10 The monoclonal antibody, PCL-2, was coupled to the activated Sepharose 4B as follows: 1 g (3.5 ml of gel) of cyanogen bromide activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, NJ) was swollen for 15 minutes in 1 mM HCl. The swollen gel was resuspended in 0.1 M
15 $NaHCO_3$, 0.5 M NaCl pH 8.3 and washed several times. The washed gel was resuspended in 11 ml of monoclonal antibody solution (PCL-2, 3.5 mg/ml in bicarbonate buffer pH 8.3) with a coupling ratio of approximately 10 mg/ml gel. Coupling was allowed to proceed for 2 h at room
20 temperature on a rotary mixer, and the gel was recovered by gentle centrifugation. The monoclonal supernatant was removed and replaced by 1 M ethanolamine in order to block any remaining sites on the Sepharose. Blocking was performed overnight at 4°C. Excess adsorbed protein was
25 removed by sequential acid and alkali washes (0.1 M acetate, 0.5 M NaCl pH 4.0; 0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3), and the coupled gel was stored in 50 mM Tris-HCl, 150 mM NaCl pH 6.5, 0.02% azide.

30 Example 6

Samples of purified recombinant transgenic protein C were compared with plasma-derived protein C and a plasma-derived activated protein C (APC) preparations. Samples were run on SDS PAGE 4-20% acrylamide gradient
35 gels under reducing conditions and silver stained for protein.

The plasma-derived material shows the presence of a heavy-chain doublet around 44 kDa (Figure 1, Lane 1). This has been reported to be due to partial occupancy of the three possible N-linked glycosylation sites on the molecule. A similar doublet, although of a slightly lower mass presumably due to some subtle change in glycosylation profile, has also been seen with the transgenic protein C. The light chain was visible around 22 kDa for both preparations. Significantly, in the case of the plasma-derived material uncleaved single-chain was clearly visible above the heavy chain doublet. Plasma-derived protein normally contained 5-10 percent of this inactive material. In contrast, the transgenic protein C contains no obvious single chain by this gel analysis. Therefore, it contains less than a few percent at most of inactive material. This most likely reflects the increased efficiency of cleavage of the modified inter-chain site. In further support of this observation no single chain was visible by direct western blot analysis of transgenic sheep milk (40387, expression level 300 µg/ml).

The purified transgenic protein C was further characterized as follows:

A. ELISA

An enzyme-linked immunosorbent assay (ELISA) for protein C was done as follows: Affinity-purified polyclonal antibody to human protein C (100 µl of 1 µg/ml in 0.1 M Na_2CO_3, pH 9.6) was added to each well of a 96-well microtiter plate, and the plates were incubated overnight at 4°C. The wells were then washed three times with phosphate buffered saline (PBS) containing 0.05% Tween-20 and incubated with 100 µl of 1% bovine serum albumin (BSA), 0.05% Tween-20 in PBS at 4°C overnight. The plates were then rinsed several times with PBS, air dried and stored at 4°C. To assay samples, 100 µl of each sample was incubated for 1 h at 37°C with a biotin-conjugated sheep polyclonal antibody to protein C (30 ng/ml) in PBS containing 1% BSA and 0.05% Tween-20. After incubation,

the wells were rinsed with PBS, and alkaline phosphatase activity was measured by the addition of 100 µl of phosphatase substrate (Sigma, St. Louis, MO) in 10%
diethanolamine, pH 9.8, containing 0.3 mM $MgCl_2$. The
5 absorbance at 405 nm was read on a microziter plate reader. Quantitation was by comparison with a standard curve using plasma-derived protein C quantitated by amino acid analysis.

10 B.   Amino-Terminal Sequencing

Amino-terminal sequencing of the transgenic material was performed to ascertain the extent of prosequence removal and to evaluate the presence of gamma-carboxylation. There were three possible N-terminal
15 sequences of protein C. These were: 1) Prosecuence which directs gamma-carboxylation and could have remained on the light chain if the first cleavage site was incompletely processed, 2) the light chain and 3) the heavy chain. N-terminal sequencing of protein C obtained from transgenic
20 milk should have contained only the latter two sequences if correct processing had occurred at both of the cleavage sites. Amino-terminal sequencing would have also been expected to reveal the presence of gamma-carboxylation in the light chain. There are nine sites of carboxylation in
25 the first twenty-nine amino acids of the light chain. On an analysis of released amino acids, the PTH-gamma carboxylic acid derivatives eluted from the HPLC column in the break-through and could therefore be analyzed. Thus, a gamma carboxylic acid showed up on the amino-terminal
30 sequence as a space rather than a glutamic acid.

The yields of amino acids in pmol released from the sequencing of approximately 27 pmol (1.4 µl) of purified transgenic protein C corresponded well to those expected for an equimolar mixture of light and heavy
35 chains, and no obvious sequence was discernible for the prosequence. Moreover, no other aberrant sequences were

detected, thus indicating a lack of inappropriate proteolytic cleavages.

As stated previously, gamma-carboxylated glutamate residues were expected to sequence as blanks using standard instrument conditions. However, sequencing protein C gives a double sequence which must be deconvoluted using knowledge of the expected light and heavy chain sequences. Normally, if the light chain alone were sequenced the gla residues at positions six and seven would appear as blanks. However when sequenced as intact protein C, the heavy chain sequence contains a glutamate residue at position six. Therefore, the only indirect confirmation of the presence of a gla residue in the light chain was the absence of glutamate at position seven which was not 'over written' by a glutamate in the heavy chain (Figure 2). Two other indirect confirmations of the presence of gamma carboxylation of the transgenic product are described below.

C. Mass Analysis of the Purified Light Chain

The protein sequence of the transgenic-derived protein C precursor had been modified with an Arg-Arg-Lys-Arg (SEQ ID NO: 20) cleavage site between the light and heavy chains to promote more efficient cleavage of the single chain to 2-chain form. Western blot analysis of the transgenic protein C milk and examination of the purified protein C on reducing gels had already confirmed that efficient cleavage had occurred. Normally during secretion, but after cleavage of the plasma-derived material, the two basic amino acids at the carboxy-terminus of the light chain are trimmed back by a basic carboxypeptide. Establishing whether the carboxy-terminus of the transgenic protein C light chain had been processed to remove the two extra basic amino acids introduced by this modification, as well as the two natural ones, was achieved by measuring the mass of the purified light chain in a quadropole instrument using on-line liquid

chromatography and electro-spray ionization. In order to achieve this, all of the cysteine residues of protein C were reduced and alkylated, and then the two chains were separated by reversed-phase chromatography.

C1. Reductive Alkylation

Because protein C is heavily cross-linked for a molecule of approximately 52 kDa, with twelve disulfide bridges (17 of the 24 cysteines involved are in the light chain), it was necessary to reductively alkylate the entire protein before attempting to separate the chains by reversed-phase chromatography. In view of the large number of cysteines in the light chain, alkylatation was done with iodoacetamide, in place of the more commonly used vinyl pyridine, to prevent the molecule from becoming excessively hydrophobic.

The transgenic protein C material (6 nmol of protein or 144 pmol of thiol) was reductively alkylated as follows: 0.5 mg of protein C (by ELISA) in 0.5 ml of TBS was added to 50 µl of 1 M Tris pH 8.0, 450 µl water, 570 mg guanidinium chloride, and 10 µl at 50 mg/ml DTT (0.3 µ mol representing a 20 fold excess of added thiol over cysteine thiol. The mixture was incubated for 2 hours at 37°C. After incubation, 20 µl at 120 mg/ml iodoacetamide (0.6 M representing a 2 fold excess over DTT on a molar basis) was added, and the mixture was incubated in the dark for one hour at 4°C. The reaction was quenched by adding 50 µl at 50 mg/ml DTT representing a 2.5 fold excess over iodoacetamide. The sample (final volume 1.5 ml) was stored at -20°C until analysis.

D. Purification of the Light Chain

Purification of protein C light chain was achieved using a large pore polystyrene column with divinyl benzene interactive groups (PLRP-S, 4000Å, 8µm, 2.1 mm ID: Polymer Laboratories, Shropshire, UK). The optimum conditions for separation of the heavy and light

chains were determined to be: solvent A (0.1% TFA) and solvent B (100% acetonitrile) at a flow of 0.5 ml/min with a detector wavelength of 215 nm and a gradient of 30 to 60% solvent B over 60 min.

Fractions were collected across the eluted peaks, and samples (10 µl) were analyzed by SDS PAGE on 4-20% gradient acrylamide gels under non-reducing conditions. The light chain (fractions 3 to 5) was completely resolved from both the heavy chain (fractions 7 to 9) and a single fraction (6) which contained a mixture of heavy chain and what appeared to be unglycosylated light chain.

A sample containing fully resolved light chain was prepared for deglycosylation by centrifugal evaporation under reduced pressure at room temperature. Deglycosylation was carried out using peptide N-glycanase (PNGase; Oxford Glycosystems, Oxford, UK). The protein sample was redissolved in 50 µl of buffer and incubated overnight with 1 unit (5 µl) PNGase, according to manufacturer's specifications.

The light chain was purified from reduced and alkylated plasma-derived protein C by the same method and deglycosylated for further analysis.

E. Analysis by Mass Spectroscopy

Samples of purified light chain were subjected to mass analysis using a liquid chromatography - electrospray interface to a Sciex Quadropole Mass Analyser (Sciex/Perkin Elmer, Toronto, CA). The LC system used a 0.5 mm ID column packed with PLRP-S 4000Å, 8µm resin (Polymer Laboratories). The solvent system contained buffer A (0.1% formic acid), buffer B (0.1% formic acid and a 5:2 (v/v) mixture of ethanol to propan-1-ol). The gradient used was from 5-60% buffer B over 35 minutes at a flow rate of 25 µl per minute. The outflow of the column was linked via a UV detector to the mass spectrometer which was run in positive-ion mode.

The purified and deglycosylated transgenic light chain was analyzed and gave a relatively weak spectrum which was reconstructed to give two components with masses of 18,911.0 and 18,971.0. The plasma light chain was also

5 analyzed and gave a stronger signal with a single major component. The spectrum of the plasma light chain was reconstructed to give a single mass of 18,970.0.

The predicted mass for the light chain carrying nine gamma-carboxy glutamic acids, one β-hydroxy aspartic

10 acid and seventeen carbamidomethyl cysteine residues and ending with Leu_{155} was 18966.9723, which is very close to the masses detected for the transgenic (18,971.0) and plasma-derived (18,970.0) light chains. The small differences in mass were well within the accuracy

15 limitations for this instrument, particularly with the LC delivery. This shows that the mass of the redirectively-alkylated and deglycosylated transgenic light chain is essentially identical to that for the plasma-derived protein C. This implies that both molecules have

20 undergone the same post-translational modifications and that the transgenic material is fully gamma carboxylated, has had all four basic amino acids trimmed back from the carboxy-terminus of the light chain and has single β hydroxy-alanine.

25

F. Activity Measurements

The activity of the transgenic protein C was compared with that of the plasma-derived material in a coagulation assay. First each sample of protein C,

30 quantitated by amino acid composition analysis, was activated by incubation with Protac, a snake venom (American Diagnostica Inc, Greenwich, CT) at a venom to protein ratio of 1 Unit Protac: 10 μg protein C for 60 minutes at 37°C. Aliquots of the activated material were

35 then compared for their ability to prolong the clotting time of protein C depleted human plasma (Diagnostic Reagents Ltd) in the presence of activated partial

thromboplastin time reagent - cephalin from rabbit brain (Sigma) and calcium using a mechanical coagulometer (Diagnostica Stago, Asmieres, FR). A comparison of clotting times with various additions of transgenic and
5   plasma-derived protein C (Figure 3) shows that the two preparations had the same anti-coagulant activity per mg of protein.

In summary, results show that the sheep-derived transgenic protein C is correctly post-translationally
10 processed, with respect to gamma-carboxylation and probably beta-hydroxylation, and has anticoagulant activity fully equivalent to a high quality purified plasma standard. The results demonstrate that the C-terminal processing of the light chain, with the modified
15 RRKR cleavage site rather than the naturally occurring KR site, has the two extra basic amino acids removed along with the natural ones.

From the foregoing, it will be appreciated that,
20 although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

25

SEQUENCE LISTING

(1) GENERAL INFORMATION:

    (i) APPLICANTS: ZymoGenetics, Inc.
                  1201 Eastlake Avenue East
                  Seattle
                  WA
                  USA
                  98102

                  PPL Therapeutics
                  Roslin
                  Edinburgh
                  Scotland
                  UK
                  EH25 9PP

    (ii) TITLE OF INVENTION: PROTEIN C PRODUCTION IN TRANSGENIC
        ANIMALS

    (iii) NUMBER OF SEQUENCES: 25

    (iv) CORRESPONDENCE ADDRESS:
        (A) ADDRESSEE: ZymoGenetics, Inc.
        (B) STREET: 1201 Eastlake Avenue East
        (C) CITY: Seattle
        (D) STATE: WA
        (E) COUNTRY: USA
        (F) ZIP: 98102

    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
        (A) NAME: Sawislak, Deborah A

     (B) REGISTRATION NUMBER: 37,438
     (C) REFERENCE/DOCKET NUMBER: 95-28PC

  (ix) TELECOMMUNICATION INFORMATION:
     (A) TELEPHONE: 206-442-6672
     (B) TELEFAX: 206-442-6678

(2) INFORMATION FOR SEQ ID NO:1:

  (i) SEQUENCE CHARACTERISTICS:
     (A) LENGTH: 11725 base pairs
     (B) TYPE: nucleic acid
     (C) STRANDEDNESS: double
     (D) TOPOLOGY: linear

  (ii) MOLECULE TYPE: DNA (genomic)

  (ix) FEATURE:
     (A) NAME/KEY: CDS
     (B) LOCATION: join(3520..3630. 5093..5117. 5210..5347. 5450
        ..5584. 8253..8395. 9269..9386. 10516..11102)

  (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
AGTGAATCTG GGCGAGTAAC ACAAAACTTG AGTGTCCTTA CCTGAAAAAT AGAGGTTAGA      60

GGGATGCTAT GTGCCATTGT GTGTGTGTGT TGGGGGTGGG GATTGGGGGT GATTTGTGAG     120

CAATTGGAGG TGAGGGTGGA GCCCAGTGCC CAGCACCTAT GCACTGGGGA CCCAAAAAGG     180

AGCATCTTCT CATGATTTTA TGTATCAGAA ATTGGGATGG CATGTCATTG GGACAGCGTC     240

TTTTTTCTTG TATGGTGGCA CATAAATACA TGTGTCTTAT AATTAATGGT ATTTTAGATT     300

TGACGAAATA TGGAATATTA CCTGTTGTGC TGATCTTGGG CAAACTATAA TATCTCTGGG     360

CAAAAATGTC CCCATCTGAA AAACAGGGAC AACGTTCCTC CCTCAGCCAG CCACTATGGG     420

GCTAAAATGA GACCACATCT GTCAAGGGTT TTGCCCTCAC CTCCCTCCCT GCTGGATGGC     480

ATCCTTGGTA GGCAGAGGTG GGCTTCGGGC AGAACAAGCC GTGCTGAGCT AGGACCAGGA     540
```

```
GTGCTAGTGC CACTGTTTGT CTATGGAGAG GGAGGCCTCA GTGCTGAGGG CCAAGCAAAT     600

ATTTGTGGTT ATGGATTAAC TCGAACTCCA GGCTGTCATG GCGGCAGGAC GGCGAACTTG     660

CAGTATCTCC ACGACCCGCC CCTGTGAGTC CCCCTCCAGG CAGGTCTATG AGGGGTGTGG     720

AGGGAGGGCT GCCCCCGGGA GAAGAGAGCT AGGTGGTGAT GAGGGCTGAA TCCTCCAGCC     780

AGGGTGCTCA ACAAGCCTGA GCTTGGGGTA AAAGGACACA AGGCCCTCCA CAGGCCAGGC     840

CTGGCAGCCA CAGTCTCAGG TCCCTTTGCC ATGCGCCTCC CTCTTTCCAG GCCAACGGTC     900

CCCAGGCCCA GGGCCATTCC AACAGACAGT TTGGAGCCCA GGACCCTCCA TTCTCCCCAC     960

CCCACTTCCA CCTTTGGGGG TGTCGGATTT GAACAAATCT CAGAAGCGGC CTCAGAGGGA    1020

GTCGGCAAGA ATGGAGAGCA GGGTCCGGTA GGGTGTGCAG AGGCCACGTG GCCTATCCAC    1080

TGGGGAGGGT TCCTTGATCT CTGGCCACCA GGGCTATCTC TGTGGCCTTT TGGAGCAACC    1140

TGGTGGTTTG GGGCAGGGGT TGAATTTCCA GGCCTAAAAC CACACAGGCC TGGCCTTGAG    1200

TCCTGGCTCT GCGAGTAATG CATGGATGTA AACATGGAGA CCCAGGACCT TGCCTCAGTC    1260

TTCCGAGTCT GGTGCCTGCA GTGTACTGAT GGTGTGAGAC CCTACTCCTG GAGGATGGGG    1320

GACAGAATCT GATCGATCCC CTGGGTTGGT GACTTCCCTG TGCAATCAAC GGAGACCAGC    1380

AAGGGTTGGA TTTTTAATAA ACCACTTAAC TCCTCCGAGT CTCAGTTTCC CCCTCTATGA    1440

AATGGGGTTG ACAGCATTAA TAACTACCTC TTGGGTGGTT GTGAGCCTTA ACTGAAGTCA    1500

TAATATCTCA TGTTTACTGA GCATGAGCTA TGTGCAAAGC CTGTTTTGAG AGCTTTATGT    1560

GGACTAACTC CTTTAATTCT CACAACACCC TTTAAGGCAC AGATACACCA CGTTATTCCA    1620

TCCATTTTAC AAATGAGGAA ACTGAGGCAT GGAGCAGTTA AGCATCTTGC CCAACATTGC    1680

CCTCCAGTAA GTGCTGGAGC TGGAATTTGC ACCGTGCAGT CTGGCTTCAT GGCCTGCCCT    1740

GTGAATCCTG TAAAAATTGT TTGAAAGACA CCATGAGTGT CCAATCAACG TTAGCTAATA    1800

TTCTCAGCCC AGTCATCAGA CCGGCAGAGG CAGCCACCCC ACTGTCCCCA GGGAGGACAC    1860

AAACATCCTG GCACCCTCTC CACTGCATTC TGGAGCTGCT TTCTAGGCAG GCAGTGTGAG    1920
```

```
CTCAGCCCCA CGTAGAGCGG GCAGCCGAGG CCTTCTGAGG CTATGTCTCT AGCGAACAAG    1980

GACCCTCAAT TCCAGCTTCC GCCTGACGGC CAGCACACAG GGACAGCCCT TTCATTCCGC    2040

TTCCACCTGG GGGTGCAGGC AGAGCAGCAG CGGGGGTAGC ACTGCCCGGA GCTCAGAAGT    2100

CCTCCTCAGA CAGGTGCCAG TGCCTCCAGA ATGTGGCAGC TCACAAGCCT CCTGCTGTTC    2160

GTGGCCACCT GGGGAATTTC CGGCACACCA GCTCCTCTTG GTAAGGCCAC CCCACCCCTA    2220

CCCCGGGACC CTTGTGGCCT CTACAAGGCC CTGGTGGCAT CTGCCCAGGC CTTCACAGCT    2280

TCCACCATCT CTCTGAGCCC TGGGTGAGGT GAGGGGCAGA TGGGAATGGC AGGAATCAAC    2340

TGACAAGTCC CAGGTAGGCC AGCTGCCAGA GTGCCACACA GGGGCTGCCA GGGCAGGCAT    2400

GCGTGATGGC AGGGAGCCCC GCGATGACCT CCTAAAGCTC CCTCCTCCAC ACGGGGATGG    2460

TCACAGAGTC CCCTGGGCCT TCCCTCTCCA CCCACTCACT CCCTCAACTG TGAAGACCCC    2520

AGGCCCAGGC TACCGTCCAC ACTATCCAGC ACAGCCTCCC CTACTCAAAT GCACACTGGC    2580

CTCATGGCTG CCCTGCCCCA ACCCCTTTCC TGGTCTCCAC AGCCAACGGG AGGAGGCCAT    2640

GATTCTTGGG GAGGTCCGCA GGCACATGGG CCCCTAAAGC CACACCAGGC TGTTGGTTTC    2700

ATTTGTGCCT TTATAGAGCT GTTTATCTGC TTGGGACCTG CACCTCCACC CTTTCCCAAG    2760

GTGCCCTCAG CTCAGGCATA CCCTCCTCTA GGATGCCTTT TCCCCCATCC CTTCTTGCTC    2820

ACACCCCCAA CTTGATCTCT CCCTCCTAAC TGTGCCCTGC ACCAAGACAG ACACTTCACA    2880

GAGCCCAGGA CACACCTGGG GACCCTTCCT GGGTGATAGG TCTGTCTATC CTCCAGGTGT    2940

CCCTGCCCAA GGGGAGAAGC ATGGGGAATA CTTGGTTGGG GGAGGAAAGG AAGACTGGGG    3000

GGATGTGTCA AGATGGGGCT GCATGTGGTG TACTGGCAGA AGAGTGAGAG GATTTAACTT    3060

GGCAGCCTTT ACAGCAGCAG CCAGGGCTTG AGTACTTATC TCTGGGCCAG GCTGTATTGG    3120

ATGTTTTACA TGACGGTCTC ATCCCCATGT TTTTGGATGA GTAAATTGAA CCTTAGAAAG    3180

GTAAAGACAC TGGCTCAAGG TCACACAGAG ATCGGGGTGG GGTTCACAGG GAGGCCTGTC    3240
```

```
CATCTCAGAG CAAGGCTTCG TCCTCCAACT GCCATCTGCT TCCTGGGGAG GAAAAGAGCA    3300
GAGGACCCCT GCGCCAAGCC ATGACCTAGA ATTAGAATGA GTCTTGAGGG GGCGGAGACA    3360
AGACCTTCCC AGGCTCTCCC AGCTCTGCTT CCTCAGACCC CCTCATGGCC CCAGCCCCTC    3420
TTAGGCCCCT CACCAAGGTG AGCTCCCCTC CCTCCAAAAC CAGACTCAGT GTTCTCCAGC    3480
AGCGAGCGTG CCCACCAGGT GCTGCGGATC CGCAAACGT GCC AAC TCC TTC CTG        3534
GAG GAG CTC CGT CAC AGC AGC CTG GAG CGG GAG TGC ATA GAG GAG ATC       3582
TGT GAC TTC GAG GAG GCC AAG GAA ATT TTC CAA AAT GTG GAT GAC ACA       3630
GTAAGGCCAC CATGGGTCCA GAGGATGAGG CTCAGGGGCG AGCTGGTAAC CAGCAGGGGC     3690
CTCGAGGAGC AGGTGGGGAC TCAATGCTGA GGCCCTCTTA GGAGTTGTGG GGGTGGCTGA     3750
GTGGAGCGAT TAGGATGCTG GCCCTATGAT GTCGGCCAGG CACATGTGAC TGCAAGAAAC     3810
AGAATTCAGG AAGAAGCTCC AGGAAAGAGT GTGGGGTGAC CCTAGGTGGG GACTCCCACA     3870
GCCACAGTGT AGGTGGTTCA GTCCACCCTC CAGCCACTGC TGAGCACCAC TGCCTCCCCG     3930
TCCCACCTCA CAAAGAGGGG ACCTAAAGAC CACCCTGCTT CCACCCATGC CTCTGCTGAT     3990
CAGGGTGTGT GTGTGACCGA AACTCACTTC TGTCCACATA AAATCGCTCA CTCTGTGCCT     4050
CACATCAAAG GGAGAAAATC TGATTGTTCA GGGGGTCGGA AGACAGGGTC TGTGTCCTAT     4110
TTGTCTAAGG GTCAGAGTCC TTTGGAGCCC CCAGAGTCCT GTGGACGTGG CCCTAGGTAG     4170
TAGGGTGAGC TTGGTAACGG GGCTGGCTTC CTGAGACAAG GCTCAGACCC GCTCTGTCCC     4230
TGGGGATCGC TTCAGCCACC AGGACCTGAA AATTGTGCAC GCCTGGGCCC CCTTCCAAGG     4290
CATCCAGGGA TGCTTTCCAG TGGAGGCTTT CAGGGCAGGA GACCCTCTGG CCTGCACCCT     4350
CTCTTGCCCT CAGCCTCCAC CTCCTTGACT GGACCCCCAT CTGGACCTCC ATCCCCACCA    4410
CCTCTTTCCC CAGTGGCCTC CCTGGCAGAC ACCACAGTGA CTTTCTGCAG GCACATATCT     4470
GATCACATCA AGTCCCCACC GTGCTCCCAC CTCACCCATG GTCTCTCAGC CCCAGCAGCC     4530
TTGGCTGGCC TCTCTGATGG AGCAGGCATC AGGCACAGGC CGTGGGTCTC AACGTGGGCT     4590
```

```
GGGTGGTCCT GGACCAGCAG CAGCCGCCGC AGCAGCAACC CTGGTACCTG GTTAGGAACG    4650

CAGACCCTCT GCCCCCATCC TCCCAACTCT GAAAAACACT GGCTTAGGGA AAGGCGCGAT    4710

GCTCAGGGGT CCCCCAAAGC CCGCAGGCAG AGGGAGTGAT GGGACTGGAA GGAGGCCGAG    4770

TGACTTGGTG AGGGATTCGG GTCCCTTGCA TGCAGAGGCT GCTGTGGGAG CGGACAGTCG    4830

CGAGAGCAGC ACTGCAGCTG CATGGGGAGA GGGTGTTGCT CCAGGGACGT GGGATGGAGG    4890

CTGGGCGCGG GCGGGTGGCG CTGGAGGGCG GGGGAGGGGC AGGGAGCACC AGCTCCTAGC    4950

AGCCAACGAC CATCGGGCGT CGATCCCTGT TTGTCTGGAA GCCCTCCCCT CCCCTGCCCG    5010

CTCACCCGCT GCCCTGCCCC ACCCGGGCGC GCCCCTCCGC ACACCGGCTG CAGGAGCCTG    5070

ACGCTGCCCG CTCTCTCCGC AG CTG GCC TTC TGG TCC AAG CAC GTC  G          5117

GTGAGTGCGT TCTAGATCCC CGGCTGGACT ACCGGCGCCC GCGCCCCTCG GGATCTCTGG    5177

CCGCTGACCC CCTACCCCGC CTTGTGTCGC AG  AC GGT GAC CAG TGC TTG GTC      5229

TTG CCC TTG GAG CAC CCG TGC GCC AGC CTG TGC TGC GGG CAC GGC ACG      5277

TGC ATC GAC GGC ATC GGC AGC TTC AGC TGC GAC TGC CGC AGC GGC TGG      5325

GAG GGC CGC TTC TGC CAG CGC  G GTGAGGGGGA GAGGTGGATG CTGGCGGGCG      5377

GCGGGGCGGG GCTGGGGCCG GGTTGGGGGC GCGGCACCAG CACCAGCTGC CCGCGCCCTC    5437

CCCTGCCCGC AG  AG GTG AGC TTC CTC AAT TGC TCT CTG GAC AAC GGC        5484

GGC TGC ACG CAT TAC TGC CTA GAG GAG GTG GGC TGG CGG CGC TGT AGC      5532

TGT GCG CCT GGC TAC AAG CTG GGG GAC GAC CTC CTG CAG TGT CAC CCC      5580

GCA  G GTGAGAAGCC CCCAATACAT CGCCCAGGAA TCACGCTGGG TGCGGGGTGG        5634

GCAGGCCCCT GACGGGCGCG GCGCGGGGGG CTCAGGAGGG TTTCTAGGGA GGGAGCGAGG    5694

AACAGAGTTG AGCCTTGGGG CAGCGGCAGA CGCGCCCAAC ACCGGGGCCA CTGTTAGCGC    5754

AATCAGCCCG GGAGCTGGGC GCGCCCTCCG CTTTCCCTGC TTCCTTTCTT CCTGGCGTCC    5814
```

```
CCGCTTCCTC CGGGCGCCCC TGCGACCTGG GGCCACCTCC TGGAGCGCAA GCCCAGTGGT    5874

GGCTCCGCTC CCCAGTCTGA GCGTATCTGG GGCGAGGCGT GCAGCGTCCT CCTCCATGTA    5934

GCCTGGCTGC GTTTTTCTCT GACGTTGTCC GGCGTGCATC GCATTTCCCT CTTTACCCCC    5994

TTGCTTCCTT GAGGAGAGAA CAGAATCCCG ATTCTGCCTT CTTCTATATT TTCCTTTTTA    6054

TGCATTTTAA TCAAATTTAT ATATGTATGA AACTTTAAAA ATCAGAGTTT TACAACTCTT    6114

ACACTTTCAG CATGCTGTTC CTTGGCATGG GTCCTTTTTT CATTCATTTT CATAAAAGGT    6174

GGACCCTTTT AATGTGGAAA TTCCTATCTT CTGCCTCTAG GGCATTTATC ACTTATTTCT    6234

TCTACAATCT CCCCTTTACT TCCTCTATTT TCTCTTTCTG GACCTCCCAT TATTCAGACC    6294

TCTTTCCTCT AGTTTTATTG TCTCTTCTAT TTCCCATCTC TTTGACTTTG TGTTTCTTT    6354

CAGGGAACTT TCTTTTTTTT CTTTTTTTTT GAGATGGAGT TTCACTCTTG TTGTCCCAGG    6414

CTGGAGTGCA ATGACGTGAT CTCAGCTCAC CACAACCTCC GCCTCCTGGA TTCAAGCGAT    6474

TCTCCTGCCG CAGCCTCCCG AGTAGCTGGG ATTACAGGCA TGCGCCACCA CGCCCAGCTA    6534

ATTTTGTGTT TTTAGTAGAG AAGGGGTTTC TCCGTGTTGG TCAAGCTGGT CTTGAACTCC    6594

TGACCTCAGG TGATCCACCT GCCTTGGCCT CCTAAAGTGC TGGGATTACA GGCGTGAGCC    6654

ACCGCGCCCA GCCTCTTTCA GGGAACTTTC TACAACTTTA TAATTCAATT CTTCTGCAGA    6714

AAAAAATTTT TGGCCAGGCT CAGTAGCTCA GACCAATAAT TCCAGCACTT TGAGAGGCTG    6774

AGGTGGGAGG ATTGCTTGAG CTTGGGAGTT TGAGACTAGC CTGGGCAACA CAGTGAGACC    6834

CTGTCTCTAT TTTTAAAAAA AGTAAAAAAA GATCTAAAAA TTTAACTTTT TATTTTGAAA    6894

TAATTAGATA TTTCCAGGAA GCTGCAAAGA AATGCCTGGT GGGCCTGTTG GCTGTGGGTT    6954

TCCTGCAAGG CCGTGGGAAG GCCCTGTCAT TGGCAGAACC CCAGATCGTG AGGGCTTTCC    7014

TTTTAGGCTG CTTTCTAAGA GGACTCCTCC AAGCTCTTGG AGGATGGAAG ACGCTCACCC    7074

ATGGTGTTCG GCCCCTCAGA GCAGGGTGGG GCAGGGGAGC TGGTGCCTGT GCAGGCTGTG    7134

GACATTTGCA TGACTCCCTG TGGTCAGCTA AGAGCACCAC TCCTTCCTGA AGCGGGGCCT    7194
```

```
GAAGTCCCTA GTCAGAGCCT CTGGTTCACC TTCTGCAGGC AGGGAGAGGG GAGTCAAGTC    7254

AGTGAGGAGG GCTTTCGCAG TTTCTCTTAC AAACTCTCAA CATGCCCTCC CACCTGCACT    7314

GCCTTCCTGG AAGCCCCACA GCCTCCTATG GTTCCGTGGT CCAGTCCTTC AGCTTCTGGG    7374

CGCCCCCATC ACGGGCTGAG ATTTTTGCTT TCCAGTCTGC CAAGTCAGTT ACTGTGTCCA    7434

TCCATCTGCT GTCAGCTTCT GGAATTGTTG CTGTTGTGCC CTTTCCATTC TTTTGTTATG    7494

ATGCAGCTCC CCTGCTGACG ACGTCCCATT GCTCTTTTAA GTCTAGATAT CTGGACTGGG    7554

CATTCAAGGC CCATTTTGAG CAGAGTCGGG CTGACCTTTC AGCCCTCAGT TCTCCATGGA    7614

GTATGCGCTC TCTTCTTGGC AGGGAGGCCT CACAAACATG CCATGCCTAT TGTAGCAGCT    7674

CTCCAAGAAT GCTCACCTCC TTCTCCCTGT AATTCCTTTC CTCTGTGAGG AGCTCAGCAG    7734

CATCCCATTA TGAGACCTTA CTAATCCCAG GGATCACCCC CAACAGCCCT GGGGTACAAT    7794

GAGCTTTTAA GAAGTTTAAC CACCTATGTA AGGAGACACA GGCAGTGGGC GATGCTGCCT    7854

GGCCTGACTC TTGCCATTGG GTGGTACTGT TTGTTGACTG ACTGACTGAC TGACTGGAGG    7914

GGGTTTGTAA TTTGTATCTC AGGGATTACC CCCAACAGCC CTGGGGTACA ATGAGCCTTC    7974

AAGAAGTTTA ACAACCTATG TAAGGACACA CAGCCAGTGG GTGATGCTGC CTGGTCTGAC    8034

TCTTGCCATT CAGTGGCACT GTTTGTTGAC TGACTGACTG ACTGACTGGC TGACTGGAGG    8094

GGGTTCATAG CTAATATTAA TGGAGTGGTC TAAGTATCAT TGGTTCCTTG AACCCTGCAC    8154

TGTGGCAAAG TGGCCCACAG GCTGGAGGAG GACCAAGACA GGAGGGCAGT CTCGGGAGGA    8214

GTGCCTGGCA GGCCCCTCAC CACCTCTGCC TACCTCAG   TG AAG TTC CCT TGT       8266

GGG AGG CCC TGG AAG CGG ATG GAG AAG AAG CGC AGT CAC CTG AAA CGA      8314

GAC ACA GAA GAC CAA GAA GAC CAA GTA GAT CCG CGG CTC ATT GAT GGG      8362

AAG ATG ACC AGG CGG GGA GAC AGC CCC TGG CAG GTGGGAGGCG AGGCAGCACC    8415

GGCTCGTCAC GTGCTGGGTC CGGGATCACT GAGTCCATCC TGGCAGCTAT GCTCAGGGTG    8475
```

```
CAGAAACCGA GAGGGAAGCG CTGCCATTGC GTTTGGGGGA TGATGAAGGT GGGGGATGCT   8535

TCAGGGAAAG ATGGACGCAA CCTGAGGGGA GAGGAGCAGC CAGGGTGGGT GAGGGCAGGG   8595

GCATGGGGGC ATGGAGGGGT CTGCAGGAGG GAGGGTTACA GTTTCTAAAA AGAGCTGGAA   8655

AGACACTGCT CTGCTGGCGG GATTTTAGGC AGAAGCCCTG CTGATGGGAG AGGGCTAGGA   8715

GGGAGGGCCG GGCCTGAGTA CCCCTCCAGC CTCCACATGG GAACTGACAC TTACTGGGTT   8775

CCCCTCTCTG CCAGGCATGG GGGAGATAGG AACCAACAAG TGGGAGTATT TGCCCTGGGG   8835

ACTCAGACTC TGCAAGGGTC AGGACCCCAA AGACCCGGCA GCCCAGTGGG ACCACAGCCA   8895

GGACGGCCCT TCAAGATAGG GGCTGAGGGA GGCCAAGGGG AACATCCAGG CAGCCTGGGG   8955

GCCACAAAGT CTTCCTGGAA GACACAAGGC CTGCCAAGCC TCTAAGGATG AGAGGAGCTC   9015

GCTGGGCGAT GTTGGTGTGG CTGAGGGTGA CTGAAACAGT ATGAACAGTG CAGGAACAGC   9075

ATGGGCAAAG GCAGGAAGAC ACCCTGGGAC AGGCTGACAC TGTAAAATGG GCAAAAATAG   9135

AAAACGCCAG AAAGGCCTAA GCCTATGCCC ATATGACCAG GGAACCCAGG AAAGTGCATA   9195

TGAAACCCAG GTGCCCTGGA CTGGAGGCTG TCAGGAGGCA GCCCTGTGAT GTCATCATCC   9255

CACCCCATTC CAG GTG GTC CTG CTG GAC TCA AAG AAG AAG CTG GCC TGC      9304

GGG GCA GTG CTC ATC CAC CCC TCC TGG GTG CTG ACA GCG GCC CAC TGC     9352

ATG GAT GAG TCC AAG AAG CTC CTT GTC AGG CTT  G GTATGGGCTG          9396

GAGCCAGGCA GAAGGGGGCT GCCAGAGGCC TGGGTAGGGG GACCAGGCAG GCTGTTCAGG   9456

TTTGGGGGAC CCCGCTCCCC AGGTGCTTAA GCAAGAGGCT TCTTGAGCTC CACAGAAGGT   9516

GTTTGGGGGG AAGAGGCCTA TGTGCCCCCA CCCTGCCCAC CCATGTACAC CCAGTATTTT   9576

GCAGTAGGGG GTTCTCTGGT GCCCTCTTCG AATCTGGGCA CAGGTACCTG CACACACATG   9636

TTTGTGAGGG GCTACACAGA CCTTCACCTC TCCACTCCCA CTCATGAGGA GCAGGCTGTG   9696

TGGGCCTCAG CACCCTTGGG TGCAGAGACC AGCAAGGCCT GGCCTCAGGG CTGTGCCTCC   9756

CACAGACTGA CAGGGATGGA GCTGTACAGA GGGAGCCCTA GCATCTGCCA AAGCCACAAG   9816
```

CTGCTTCCCT AGCAGGCTGG GGGCTCCTAT GCATTGGCCC CGATCTATGG CAATTTCTGG   9876

AGGGGGGGTC TGGCTCAACT CTTTATGCCA AAAAGAAGGC AAAGCATATT GAGAAAGGCC   9936

AAATTCACAT TTCCTACAGC ATAATCTATG CCAGTGGCCC CGTGGGGCTT GGCTTAGAAT   9996

TCCCAGGTGC TCTTCCCAGG GAACCATCAG TCTGGACTGA GAGGACCTTC TCTCTCAGGT   10056

GGGACCCGGC CCTGTCCTCC CTGGCAGTGC CGTGTTCTGG GGGTCCTCCT CTCTGGGTCT   10116

CACTGCCCCT GGGGTCTCTC CAGCTACCTT TGCTCCATGT TCCTTTGTGG CTCTGGTCTG   10176

TGTCTGGGGT TTCCAGGGGT CTCGGGCTTC CCTGCTGCCC ATTCCTTCTC TGGTCTCACG   10236

GCTCCGTGAC TCCTGAAAAC CAACCAGCAT CCTACCCCTT TGGATTGACA CCTGTTGGCC   10296

ACTCCTTCTG GCAGGAAAAG TCACCGTTGA TAGGGTTCCA CGGCATAGAC AGGTGGCTCC   10356

GCGCCAGTGC CTGGGACGTG TGGGTGCACA GTCTCCGGGT GAACCTTCTT CAGGCCCTCT   10416

CCCAGGCCTG CAGGGGCACA GCAGTGGGTG GGCCTCAGGA AAGTGCCACT GGGGAGAGGC   10476

TCCCCGCAGC CCACTCTGAC TGTGCCCTCT GCCCTGCAG GA GAG TAT GAC CTG   10529

CGG CGC TGG GAG AAG TGG GAG CTG GAC CTG GAC ATC AAG GAG GTC TTC   10577

GTC CAC CCC AAC TAC AGC AAG AGC ACC ACC GAC AAT GAC ATC GCA CTG   10625

CTG CAC CTG GCC CAG CCC GCC ACC CTC TCG CAG ACC ATA GTG CCC ATC   10673

TGC CTC CCG GAC AGC GGC CTT GCA GAG CGC GAG CTC AAT CAG GCC GGC   10721

CAG GAG ACC CTC GTG ACG GGC TGG GGC TAC CAC AGC AGC CGA GAG AAG   10769

GAG GCC AAG AGA AAC CGC ACC TTC GTC CTC AAC TTC ATC AAG ATT CCC   10817

GTG GTC CCG CAC AAT GAG TGC AGC GAG GTC ATG AGC AAC ATG GTG TCT   10865

GAG AAC ATG CTG TGT GCG GGC ATC CTC GGG GAC CGG CAG GAT GCC TGC   10913

GAG GGC GAC AGT GGG GGG CCC ATG GTC GCC TCC TTC CAC GGC ACC TGG   10961

TTC CTG GTG GGC CTG GTG AGC TGG GGT GAG GGC TGT GGG CTC CTT CAC   11009

```
AAC TAC GGC GTT TAC ACC AAA GTC AGC CGC TAC CTC GAC TGG ATC CAT      11057

GGG CAC ATC AGA GAC AAG GAA GCC CCC CAG AAG AGC TGG GCA CCT          11102

TAGCGACCCT CCCTGCAGGG CTGGGCTTTT GCATGGCAAT GGATGGGACA TTAAAGGGAC   11162

ATGTAACAAG CACACCGGCC TGCTGTTCTG TCCTTCCATC CCTCTTTTGG GCTCTTCTGG   11222

AGGGAAGTAA CATTTACTGA GCACCTGTTG TATGTCACAT GCCTTATGAA TAGAATCTTA   11282

ACTCCTAGAG CAACTCTGTG GGGTGGGGAG GAGCAGATCC AAGTTTTGCG GGGTCTAAAG   11342

CTGTGTGTGT TGAGGGGGAT ACTCTGTTTA TGAAAAAGAA TAAAAAACAC AACCACGAAG   11402

CCACTAGAGC CTTTTCCAGG GCTTTGGGAA GAGCCTGTGC AAGCCGGGGA TGCTGAAGGT   11462

GAGGCTTGAC CAGCTTTCCA GCTAGCCCAG CTATGAGGTA GACATGTTTA GCTCATATCA   11522

CAGAGGAGGA AACTGAGGGG TCTGAAAGGT TTACATGGTG GAGCCAGGAT TCAAATCTAG   11582

GTCTGACTCC AAAACCCAGG TGCTTTTTTC TGTTCTCCAC TGTCCTGGAG GACAGCTGTT   11642

TCGACGGTGC TCAGTGTGGA GGCCACTATT AGCTCTGTAG GGAAGCAGCC AGAGACCCAG   11702

AAAGTGTTGG TTCAGCCCAG AAT                                           11725
```

(2) INFORMATION FOR SEQ ID NO:2:

   (i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 460 amino acids
      (B) TYPE: amino acid
      (D) TOPOLOGY: linear

   (ii) MOLECULE TYPE: protein

   (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile
1               5                   10                  15

Ser Gly Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg
            20                  25                  30
```

```
Ala His Gln Val Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu
         35              40                    45

Glu Leu Arg His Ser Ser Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys
    50                  55                60

Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln Asn Val Asp Asp Thr Leu
65              70                  75                      80

Ala Phe Trp Ser Lys His Val Asp Gly Asp Gln Cys Leu Val Leu Pro
             85                  90                      95

Leu Glu His Pro Cys Ala Ser Leu Cys Cys Gly His Gly Thr Cys Ile
             100                105                110

Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp Glu Gly
             115                120                125

Arg Phe Cys Gln Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn
    130                135                140

Gly Gly Cys Thr His Tyr Cys Leu Glu Glu Val Gly Trp Arg Arg Cys
145                150                155                160

Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp Asp Leu Leu Gln Cys His
                 165                170                175

Pro Ala Val Lys Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys
                 180                185                190

Lys Arg Ser His Leu Lys Arg Asp Thr Glu Asp Gln Glu Asp Gln Val
    195                200                205

Asp Pro Arg Leu Ile Asp Gly Lys Met Thr Arg Arg Gly Asp Ser Pro
    210                215                220

Trp Gln Val Val Leu Leu Asp Ser Lys Lys Lys Leu Ala Cys Gly Ala
225                230                235                240

Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala His Cys Met Asp
                 245                250                255

Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu Arg Arg
             260                265                270
```

```
Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Lys Glu Val Phe Val His
        275                 280                 285

Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Asp Ile Ala Leu Leu His
        290             295                 300

Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu
305                 310                 315                 320

Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu Asn Gln Ala Gly Gln Glu
            325                 330                 335

Thr Leu Val Thr Gly Trp Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
            340                 345                 350

Lys Arg Asn Arg Thr Phe Val Leu Asn Phe Ile Lys Ile Pro Val Val
            355                 360                 365

Pro His Asn Glu Cys Ser Glu Val Met Ser Asn Met Val Ser Glu Asn
        370             375                 380

Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp Ala Cys Glu Gly
385                 390                 395                 400

Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp Phe Leu
                405                 410                 415

Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr
            420                 425                 430

Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His
    435                 440                 445

Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala
    450                 455                 460
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1386 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(ix) FEATURE:
    (A) NAME/KEY: CDS
    (B) LOCATION: 1..1380

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
ATG TGG CAG CTC ACA AGC CTC CTG CTG TTC GTG GCC ACC TGG GGA ATT      48
Met Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile
1               5                   10                  15

TCC GGC ACA CCA GCT CCT CTT GAC TCA GTG TTC TCC AGC AGC GAG CGT      96
Ser Gly Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg
            20                  25                  30

GCC CAC CAG GTG CTG CGG ATC CGC AAA CGT GCC AAC TCC TTC CTG GAG     144
Ala His Gln Val Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu
        35                  40                  45

GAG CTC CGT CAC AGC AGC CTG GAG CGG GAG TGC ATA GAG GAG ATC TGT     192
Glu Leu Arg His Ser Ser Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys
    50                  55                  60

GAC TTC GAG GAG GCC AAG GAA ATT TTC CAA AAT GTG GAT GAC ACA CTG     240
Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln Asn Val Asp Asp Thr Leu
65                  70                  75                  80

GCC TTC TGG TCC AAG CAC GTC GAC GGT GAC CAG TGC TTG GTC TTG CCC     288
Ala Phe Trp Ser Lys His Val Asp Gly Asp Gln Cys Leu Val Leu Pro
                85                  90                  95

TTG GAG CAC CCG TGC GCC AGC CTG TGC TGC GGG CAC GGC ACG TGC ATC     336
Leu Glu His Pro Cys Ala Ser Leu Cys Cys Gly His Gly Thr Cys Ile
            100                 105                 110

GAC GGC ATC GGC AGC TTC AGC TGC GAC TGC CGC AGC GGC TGG GAG GGC     384
Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp Glu Gly
        115                 120                 125

CGC TTC TGC CAG CGC GAG GTG AGC TTC CTC AAT TGC TCT CTG GAC AAC     432
Arg Phe Cys Gln Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn
    130                 135                 140
```

```
GGC GGC TGC ACG CAT TAC TGC CTA GAG GAG GTG GGC TGG CGG CGC TGT
Gly Gly Cys Thr His Tyr Cys Leu Glu Glu Val Gly Trp Arg Arg Cys      480
145                 150                 155                 160

AGC TGT GCG CCT GGC TAC AAG CTG GGG GAC GAC CTC CTG CAG TGT CAC
Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp Asp Leu Leu Gln Cys His      528
            165                 170                 175

CCC GCA GTG AAG TTC CCT TGT GGG AGG CCC TGG AAG CGG ATG GAG AAG
Pro Ala Val Lys Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys      576
            180                 185                 190

AAG CGC AGT CAC CTG AAA CGA GAC ACA GAA GAC CAA GAA GAC CAA GTA
Lys Arg Ser His Leu Lys Arg Asp Thr Glu Asp Gln Glu Asp Gln Val      624
            195                 200                 205

GAT CCG CGG CTC ATT GAT GGG AAG ATG ACC AGG CGG GGA GAC AGC CCC
Asp Pro Arg Leu Ile Asp Gly Lys Met Thr Arg Arg Gly Asp Ser Pro      672
    210                 215                 220

TGG CAG GTG GTC CTG CTG GAC TCA AAG AAG AAG CTG GCC TGC GGG GCA
Trp Gln Val Val Leu Leu Asp Ser Lys Lys Lys Leu Ala Cys Gly Ala      720
225                 230                 235                 240

GTG CTC ATC CAC CCC TCC TGG GTG CTG ACA GCG GCC CAC TGC ATG GAT
Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala His Cys Met Asp      768
                245                 250                 255

GAG TCC AAG AAG CTC CTT GTC AGG CTT GGA GAG TAT GAC CTG CGG CGC
Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu Arg Arg      816
            260                 265                 270

TGG GAG AAG TGG GAG CTG GAC CTG GAC ATC AAG GAG GTC TTC GTC CAC
Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Lys Glu Val Phe Val His      864
            275                 280                 285

CCC AAC TAC AGC AAG AGC ACC ACC GAC AAT GAC ATC GCA CTG CTG CAC
Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Asp Ile Ala Leu Leu His      912
            290                 295                 300

CTG GCC CAG CCC GCC ACC CTC TCG CAG ACC ATA GTG CCC ATC TGC CTC
Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu      960
305                 310                 315                 320
```

```
CCG GAC AGC GGC CTT GCA GAG CGC GAG CTC AAT CAG GCC GGC CAG GAG        1008
Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu Asn Gln Ala Gly Gln Glu
            325             330             335

ACC CTC GTG ACG GGC TGG GGC TAC CAC AGC AGC CGA GAG AAG GAG GCC        1056
Thr Leu Val Thr Gly Trp Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
            340             345             350

AAG AGA AAC CGC ACC TTC GTC CTC AAC TTC ATC AAG ATT CCC GTG GTC        1104
Lys Arg Asn Arg Thr Phe Val Leu Asn Phe Ile Lys Ile Pro Val Val
            355             360             365

CCG CAC AAT GAG TGC AGC GAG GTC ATG AGC AAC ATG GTG TCT GAG AAC        1152
Pro His Asn Glu Cys Ser Glu Val Met Ser Asn Met Val Ser Glu Asn
            370             375             380

ATG CTG TGT GCG GGC ATC CTC GGG GAC CGG CAG GAT GCC TGC GAG GGC        1200
Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp Ala Cys Glu Gly
385             390             395             400

GAC AGT GGG GGG CCC ATG GTC GCC TCC TTC CAC GGC ACC TGG TTC CTG        1248
Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp Phe Leu
            405             410             415

GTG GGC CTG GTG AGC TGG GGT GAG GGC TGT GGG CTC CTT CAC AAC TAC        1296
Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr
            420             425             430

GGC GTT TAC ACC AAA GTC AGC CGC TAC CTC GAC TGG ATC CAT GGG CAC        1344
Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His
            435             440             445

ATC AGA GAC AAG GAA GCC CCC CAG AAG AGC TGG GCA CCTTAG             1386
Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala
            450             455             460
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 460 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Met Trp Gln Leu Thr Ser Leu Leu Leu Phe Val Ala Thr Trp Gly Ile
 1               5                  10                  15

Ser Gly Thr Pro Ala Pro Leu Asp Ser Val Phe Ser Ser Ser Glu Arg
                20              25                  30

Ala His Gln Val Leu Arg Ile Arg Lys Arg Ala Asn Ser Phe Leu Glu
            35                  40                  45

Glu Leu Arg His Ser Ser Leu Glu Arg Glu Cys Ile Glu Glu Ile Cys
        50                  55                  60

Asp Phe Glu Glu Ala Lys Glu Ile Phe Gln Asn Val Asp Asp Thr Leu
65                  70                  75                  80

Ala Phe Trp Ser Lys His Val Asp Gly Asp Gln Cys Leu Val Leu Pro
                85                  90                  95

Leu Glu His Pro Cys Ala Ser Leu Cys Cys Gly His Gly Thr Cys Ile
            100             105                 110

Asp Gly Ile Gly Ser Phe Ser Cys Asp Cys Arg Ser Gly Trp Glu Gly
        115             120                 125

Arg Phe Cys Gln Arg Glu Val Ser Phe Leu Asn Cys Ser Leu Asp Asn
    130             135                 140

Gly Gly Cys Thr His Tyr Cys Leu Glu Glu Val Gly Trp Arg Arg Cys
145             150                 155                 160

Ser Cys Ala Pro Gly Tyr Lys Leu Gly Asp Asp Leu Leu Gln Cys His
                165                 170                 175

Pro Ala Val Lys Phe Pro Cys Gly Arg Pro Trp Lys Arg Met Glu Lys
            180             185                 190

Lys Arg Ser His Leu Lys Arg Asp Thr Glu Asp Gln Glu Asp Gln Val
        195                 200                 205

Asp Pro Arg Leu Ile Asp Gly Lys Met Thr Arg Arg Gly Asp Ser Pro
    210                 215                 220
```

Trp Gln Val Val Leu Leu Asp Ser Lys Lys Lys Leu Ala Cys Gly Ala
225   230   235   240

Val Leu Ile His Pro Ser Trp Val Leu Thr Ala Ala His Cys Met Asp
                    245   250   255

Glu Ser Lys Lys Leu Leu Val Arg Leu Gly Glu Tyr Asp Leu Arg Arg
                260   265   270

Trp Glu Lys Trp Glu Leu Asp Leu Asp Ile Lys Glu Val Phe Val His
            275   280   285

Pro Asn Tyr Ser Lys Ser Thr Thr Asp Asn Asp Ile Ala Leu Leu His
        290   295   300

Leu Ala Gln Pro Ala Thr Leu Ser Gln Thr Ile Val Pro Ile Cys Leu
305   310   315   320

Pro Asp Ser Gly Leu Ala Glu Arg Glu Leu Asn Gln Ala Gly Gln Glu
                325   330   335

Thr Leu Val Thr Gly Trp Gly Tyr His Ser Ser Arg Glu Lys Glu Ala
            340   345   350

Lys Arg Asn Arg Thr Phe Val Leu Asn Phe Ile Lys Ile Pro Val Val
        355   360   365

Pro His Asn Glu Cys Ser Glu Val Met Ser Asn Met Val Ser Glu Asn
    370   375   380

Met Leu Cys Ala Gly Ile Leu Gly Asp Arg Gln Asp Ala Cys Glu Gly
385   390   395   400

Asp Ser Gly Gly Pro Met Val Ala Ser Phe His Gly Thr Trp Phe Leu
                405   410   415

Val Gly Leu Val Ser Trp Gly Glu Gly Cys Gly Leu Leu His Asn Tyr
            420   425   430

Gly Val Tyr Thr Lys Val Ser Arg Tyr Leu Asp Trp Ile His Gly His
            435   440   445

Ile Arg Asp Lys Glu Ala Pro Gln Lys Ser Trp Ala
    450   455   460

(2) INFORMATION FOR SEQ ID NO:5:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10807 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
ACGCGTGTCG ACCTGCAGGT CAACGGATCT CTGTGTCTGT TTTCATGTTA GTACCACACT      60
GTTTTGGTGG CTGTAGCTTT CAGCTACAGT CTGAAGTCAT AAAGCCTGGT ACCTCCAGCT     120
CTGTTCTCTC TCAAGATTGT GTTCTGCTGT TTGGGTCTTT AGTGTCTCCA CACAATTTTT     180
AGAATTGTTT GTTCTAGTTC TGTGAAAAAT GATGCTGGTA TTTTGATAAG GATTGCATTG     240
AATCTGTAAA GCTACAGATA TAGTCATTGG GTAGTACAGT CACTTTAACA ATATTAACTC     300
TTCACATCTG TGAGCATGAT ATATTTTCCC CCTCTATATC ATCTTCAATT CCTCCTATCA     360
GTTTCTTTCA TTGCAGTTTT CTGAGTACAG GTCTTACACC TCCTTGGTTA GAGTCATTCC     420
TCAGTATTTT ATTCCTTTGA TACAATTGTG AATGAGGTAA TTTTCTTAGT TTCTCTTTCT     480
GATAGCTCAT TGTTAGTGTA TATATAGAAA AGCAACAGAT TTCTATGTAT TAATTTTGTA     540
TCCTGCAACA GATTTCTATG TATTAATTTT GTATCCTGCT ACTTTACGGA ATTCACTTAT     600
TAGCTTTTTG GTGACATCTT GAGGATTTTC TGAAGAAAAT GGCATGGTAT GGTACGACAA     660
GGTGTCATGT CATCTGCAAA CAGTGGCAGT TTTCCTTCTT CCCTTCCAAC CTGGATTTCT     720
TTGATTTCTT TCTGTCTGAG TACGACTAGG ATTCCCAATA CTATACCGAA TAAAAGTGGC     780
AAGAGTGGAC ATCCTTGTCT TATTTTTCTG ACCTTAGAGG AAATGCTTTC AGTTTTTCAC     840
CATTAATTAT AATGTTTACT GTGGGCTTGT CATATGTGGC CTTCATTATA TGGACGTCTA     900
TTCCCTCTAT ACCCACCTTG TTGAGAGTTT TTATCATAAA AGTATGTTGA ATTTTGTCAA     960
AAGTTTTTCC TGCATCTATT GAGATGATTT TTACTCTTCA ATTCATTAAT GATTTTTATT    1020
```

```
CTTCATTTTG TTAATGATTT CCATTCTTCA ATTTGTTAAC GTGGTATATC ACATTGATTG    1080

ATTTGTGGAT ACCTTTGTAT CCCTGGGATA AACCTCACTT GATCATGAGC TTTCAATGTA    1140

TTTTTGAATT CACTTTGCTA ATATTCTGTT GGGTATTTTT GCATCTCTAT TCATCAATGA    1200

TATTGGCCTA AGAAAGGTTT TGTCTGGTTT TAGTATCAGG GTGATGCTGG CCTCATAGAG    1260

AGAGTTTAGA AGCATTTCCT CCTCTTTGAT TTTTCGGAAT AGTTTGAGTA GGATAGGTAT    1320

TAACTCTTCT TTAAATGTTT GGGGACTTCC CTGGTGAGCC GGTGGTTGAG AATCCGCCTC    1380

AGGGATGTGG GTTTGATCCC TGGTCAGGGA ACCATTAATA AGATCCCACA TGCTGCAGGC    1440

AACAAGCCCC CAAGCTGCAA CCACTGAGCT GCAACCGCTG CAGTGCCCAC AGGCCACGAC    1500

CAGAGAAAGC CCACATACAG CAGGGAAGAC CCAGCACAAC CGGAAAAAGG AGTTTGGTGG    1560

AATACAGCTG TGAAGCCGTC TGGTCCTGGA CTCCTGCTTG AGGGAATTTT TTAAAAATTA    1620

TTGATTCAAT TTCATTACTG GTAACTGGTC TGTTCATATT TTCTATTTCT TCCGGGTTCA    1680

GTCTTGGGAG ATTGTACATG CCTAGGAATG TGTCCGTTTC TTCTAGGTTG TCCATTTTAT    1740

TGGACATGCA TGGGAGCACA CAGCACCGAC CAGCGAGACT CATGCTGGCT TCCTGGGGCC    1800

AGGCTGGGGC CCCAAGCAGC ATGGCATCCT AGAGTGTGTG AAAGCCCACT GACCCTGCCC    1860

AGCCCCACAA TTTCATTCTG AGAAGTGATT CCTTGCTTCT GCACTTACAG GCCCAGGATC    1920

TGACCTGCTT CTGAGGAGCA GGGGTTTTGG CAGGACGGGG AGATGCTGAG AGCCGACGGG    1980

GGTCCAGGTC CCCTCCCAGG CCCCCCTGTC TGGGGCAGCC CTTGGGAAAG ATTGCCCCAG    2040

TCTCCCTCCT ACAGTGGTCA GTCCCAGCTG CCCCAGGCCA GAGCTGCTTT ATTTCCGTCT    2100

CTCTCTCTGG ATGGTATTCT CTGGAAGCTG AAGGTTCCTG AAGTTATGAA TAGCTTTGCC    2160

CTGAAGGGCA TGGTTTGTGG TCACGGTTCA CAGGAACTTG GGAGACCCTG CAGCTCAGAC    2220

GTCCCGAGAT TGGTGGCACC CAGATTTCCT AAGCTCGCTG GGGAACAGGG CGCTTGTTTC    2280

TCCCTGGCTG ACCTCCCTCC TCCCTGCATC ACCCAGTTCT GAAAGCAGAG CGGTGCTGGG    2340
```

```
GTCACAGCCT CTCGCATCTA ACGCCGGTGT CCAAACCACC CGTGCTGGTG TTCGGGGGGC    2400

TACCTATGGG GAAGGGCTTC TCACTGCAGT GGTGCCCCCC GTCCCTCTG AGATCAGAAG     2460

TCCCAGTCCG GACGTCAAAC AGGCCGAGCT CCCTCCAGAG GCTCCAGGGA GGGATCCTTG    2520

CCCCCCCGCT GCTGCCTCCA GCTCCTGGTG CCGCACCCTT GAGCCTGATC TTGTAGACGC    2580

CTCAGTCTAG TCTCTGCCTC CGTGTTCACA CGCCTTCTCC CCATGTCCCC TCCGTGTCCC    2640

CGTTTTCTCT CACAAGGACA CCGGACATTA GATTAGCCCC TGTTCCAGCC TCACCTGAAC    2700

AGCTCACATC TGTAAAGACC TAGATTCCAA ACAAGATTCC AACCTGAAGT TCCCGGTGGA    2760

TGTGAGTTCT GGGGCGACAT CCTTCAACCC CATCACAGCT TGCAGTTCAT CGCAAAACAT    2820

GGAACCTGGG GTTTATCGTA AAACCCAGGT TCTTCATGAA ACACTGAGCT TCGAGGCTTG    2880

TTGCAAGAAT TAAAGGTGCT AATACAGATC AGGGCAAGGA CTGAAGCTGG CTAAGCCTCC    2940

TCTTTCCATC ACAGGAAAGG GGGGCCTGGG GGCGGCTGGA GGTCTGCTCC CGTGAGTGAG    3000

CTCTTTCCTG CTACAGTCAC CAACAGTCTC TCTGGGAAGG AAACCAGAGG CCAGAGAGCA    3060

AGCCGGAGCT AGTTTAGGAG ACCCCTGAAC CTCCACCCAA GATGCTGACC AGCCAGCGGG    3120

CCCCCTGGAA AGACCCTACA GTTCAGGGGG GAAGAGGGGC TGACCCGCCA GGTCCCTGCT    3180

ATCAGGAGAC ATCCCCGCTA TCAGGAGATT CCCCCACCTT GCTCCCGTTC CCCTATCCCA    3240

ATACGCCCAC CCCACCCCTG TGATGAGCAG TTTAGTCACT TAGAATGTCA ACTGAAGGCT    3300

TTTGCATCCC CTTTGCCAGA GGCACAAGGC ACCCACAGCC TGCTGGGTAC CGACGCCCAT    3360

GTGGATTCAG CCAGGAGGCC TGTCCTGCAC CCTCCCTGCT CGGGCCCCCT CTGTGCTCAG    3420

CAACACACCC AGCACCAGCA TTCCCGCTGC TCCTGAGGTC TGCAGGCAGC TCGCTGTAGC    3480

CTGAGCGGTG TGGAGGGAAG TGTCCTGGGA GATTTAAAAT GTGAGAGGCG GGAGGTGGGA    3540

GGTTGGGCCC TGTGGGCCTG CCCATCCCAC GTGCCTGCAT TAGCCCCAGT GCTGCTCAGC    3600

CGTGCCCCCG CCGCAGGGGT CAGGTCACTT TCCCGTCCTG GGGTTATTAT GACTCTGTC     3660

ATTGCCATTG CCATTTTTGC TACCCTAACT GGGCAGCAGG TGCTTGCAGA GCCCTCGATA    3720
```

```
CCGACCAGGT CCTCCCTCGG AGCTCGACCT GAACCCCATG TCACCCTTGC CCCAGCCTGC    3780

AGAGGGTGGG TGACTGCAGA GATCCCTTCA CCCAAGGCCA CGGTCACATG GTTTGGAGGA    3840

GCTGGTGCCC AAGGCAGAGG CCACCCTCCA GGACACACCT GTCCCCAGTG CTGGCTCTGA    3900

CCTGTCCTTG TCTAAGAGGC TGACCCCGGA AGTGTTCCTG GCACTGGCAG CCAGCCTGGA    3960

CCCAGAGTCC AGACACCCAC CTGTGCCCCC GCTTCTGGGG TCTACCAGGA ACCGTCTAGG    4020

CCCAGAGGGG ACTTCCTGCT TGGCCTTGGA TGGAAGAAGG CCTCCTATTG TCCTCGTAGA    4080

GGAAGCCACC CCGGGGCCTG AGGATGAGCC AAGTGGGATT CCGGGAACCG CGTGGCTGGG    4140

GGCCCAGCCC GGGCTGGCTG GCCTGCATGC CTCCTGTATA AGGCCCCAAG CCTGCTGTCT    4200

CAGCCCTCCA CTCCCTGCAG AGCTCAGAAG CACGACCCCA GGGATATCCC TGCAGCCATG    4260

AAGTGCCTCC TGCTTGCCCT GGGCCTGGCC CTCGCCTGTG GCGTCCAGGC CATCATCGTC    4320

ACCCAGACCA TGAAAGGCCT GGACATCCAG AAGGTTCGAG GGTTGGCCGG GTGGGTGAGT    4380

TGCAGGGCGG GCAGGGGAGC TGGGCCTCAG AGAGCCAAGA GAGGCTGTGA CGTTGGGTTC    4440

CCATCAGTCA GCTAGGGCCA CCTGACAAAT CCCCGCTGGG GCAGCTTCAA CCAGGCGTTC    4500

ACTGTCTTGC ATTCTGGAGG CTGGAAGCCC AAGATCCAGG TGTTGGCAGG GCTGGCTTCT    4560

CCTGCGGCCG CTCTCTGGGG AGCAGACGGC CGTCTTCTCC AGTCCTCTGC GCGCCCTGAT    4620

TTCCTCTTCC TGTGAGGCCA CCAGGCCTGC TGGAAACACG CCTGCCTGCG CAGCTTCACA    4680

CGACCTTTGT CATCTCTTTA AAGGCCATGT CTCCAGAGTC ATGTGTTGAA GTTCTGGGGG    4740

TTAGTGGGAC ACAGTTCAGC CCCTAAAAGA GTCTCTCTGC CCCTCAAATT TTCCCCACCT    4800

CCAGCCATGT CTCCCCAAGA TCCAAATGTT GCTACATGTG GGGGGGCTCA TCTGGGTCCC    4860

TCTTTGGGTT CAGTGTGAGT CTGGGGAGAG CATTCCCCAG GGTGCAGAGT TGGGGGGAGT    4920

ATCTCAGGGC TGCCCAGGCC GGGGTGGGAC AGAGAGCCCA CTGTGGGGCT GGGGGCCCCT    4980

TCCCACCCCC AGAGTGCAAC TCAAGGTCCC TCTCCAGGTG GCGGGGACTT GGCACTCCTT    5040
```

```
GGCTATGGCG GCCAGCGACA TCTCCCTGCT GGATGCCCAG AGTGCCCCCC TGAGAGTGTA      5100
CGTGGAGGAG CTGAAGCCCA CCCCCGAGGG CAACCTGGAG ATCCTGCTGC AGAAATGGTG      5160
GGCGTCTCTC CCCAACATGG AACCCCCACT CCCCAGGGCT GTGGACCCCC CGGGCGGTGG      5220
GGTGCAGGAG GGACCAGGGC CCCAGGGCTG GGGAAGAGGG CTCAGAGTTT ACTGCTACCC      5280
GGCGCTCCAC CCAAGGCTGC CCACCCAGGG CTTTTTTTTT TTTTAAACTT TTATTAATTT      5340
GATGCTTCAG AACATCATCA AACAAATGAA CATAAAACAT TCATTTTTGT TTACTTGGAA      5400
GGGGAGATAA AATCCTCTGA AGTGGAAATG CATAGCAAAG ATACATACAA TGAGGCAGGT      5460
ATTCTGAATT CCCTGTTAGT CTGAGGATTA CAAGTGTATT TGAGCAACAG AGAGACATTT      5520
TCATCATTTC TAGTCTGAAC ACCTCAGTAT CTAAAATGAA CAAGAAGTCC TGGAAACGAA      5580
GCAGTGTGGG GATAGGCCCG TGTGAAGGCT GCTGGGAGGC AGCAGACCTG GGTCTTCGGG      5640
CTCAAGCAGT TCCCGCTACC AGCCCTGTCC ACCTCAGACG GGGGTCAGGG TGCAGGAGAG      5700
AGCTGGATGG GTGTGGGGGC AGAGATGGGG ACCTGAACCC CAGGGCTGCC TTTTGGGGGT      5760
GCCTGTGGTC AAGGCTCTCC CTGACCTTTT CTCTCTGGCT TCATCTGACT TCTCCTGGCC      5820
CATCCACCCG GTCCCCTGTG GCCTGAGGTG ACAGTGAGTG CGCCGAGGCT AGTTGGCCAG      5880
CTGGCTCCTA TGCCCATGCC ACCCCCCTCC AGCCCTCCTG GGCCAGCTTC TGCCCCTGGC      5940
CCTCAGTTCA TCCTGATGAA AATGGTCCAT GCCAATGGCT CAGAAAGCAG CTGTCTTTCA      6000
GGGAGAACGG CGAGTGTGCT CAGAAGAAGA TTATTGCAGA AAAAACCAAG ATCCCTGCGG      6060
TGTTCAAGAT CGATGGTGAG TCCGGGTCCC TGGGGGACAC CCACCACCCC CGCCCCCGGG      6120
GACTGTGGAC AGGTTCAGGG GGCTGGCGTC GGGCCCTGGG ATGCTAAGGG ACTGGTGGTG      6180
ATGAAGACAC TGCCTTGACA CCTGCTTCAC TTGCCTCCCC TGCCACCTGC CCGGGGCCTT      6240
GGGGCGGTGG CCATGGGCAG GTCCCGGCTG GCGGGCTAAC CCACCAGGGT GACACCCGAG      6300
CTCTCTTTGC TGGGGGGCGG GCGGTGCTCT GGGCCCTCAG GCTGAGCTCA GGAGGCACCT      6360
GTGCCCTCCC AGGGGTAACC GAGAGCCGTT GCCCACTCCA GGGGCCCAGG TGCCCCACGA      6420
```

```
CCCCAGCCCG CTCCACAGCT CCTTCATCTC CTGGAGACAA ACTCTGTCCG CCCTCGCTCA   6480

TTCACTTGTT CGTCCTAAAT CCGAGATGAT AAAGCTTCGA GGGGGGGGTTG GGGTTCCATC   6540

AGGGCTGCCC TTCCGCCGGG CAGCCTGGGC CACATCTGCC CTTGGCCCCC TCAGGACTCA   6600

CTCTGACTGG AGGCCCTGCA CTGACTGACG CCAGGGTGCC CAGCCCAGGG TCTCTGGCGC   6660

CATCCAGCTG CACTGGGTTT GGGTGCTGGT CCTGCCCCCA AGCTGCCCGG ACACCACAGG   6720

CAGCCGGGGC TGCCCACTGG CCTCGGTCAG GGTGAGCCCC AGCTGCCCCC GCTCAGGGCT   6780

TGCCCCGACA ATGACCCCAT CCTCAGGACG CACCCCCCTT CCCTTGCTGG GCAGTGTCCA   6840

GCCCCACCCG AGATCGGGGG AAGCCCTATT TCTTGACAAC TCCAGTCCCT GGGGGAGGGG   6900

GCCTCAGACT GAGTGGTGAG TGTTCCCAAG TCCAGGAGGT GGTGGAGGGT CCTGGCGGAT   6960

CCAGAGTTGA CAGTGAGGGC TTCCTGGGCC CCATGCGCCT GGCAGTGGCA GCAGGGAAGA   7020

GGAAGCACCA TTTCAGGGGT GGGGGATGCC AGAGGCGCTC CCCACCCCGT CTTCGCCGGG   7080

TGGTGACCCC GGGGGAGCCC CGCTGGTCGT GGAGGGTGCT GGGGGCTGAC TAGCAACCCC   7140

TCCCCCCCCG TTGGAACTCA CTTTTCTCCC GTCTTGACCG CGTCCAGCCT TGAATGAGAA   7200

CAAAGTCCTT GTGCTGGACA CCGACTACAA AAAGTACCTG CTCTTCTGCA TGGAAAACAG   7260

TGCTGAGCCC GAGCAAAGCC TGGCCTGCCA GTGCCTGGGT GGGTGCCAAC CCTGGCTGCC   7320

CAGGGAGACC AGCTGCGTGG TCCTTGCTGC AACAGGGGGT GGGGGGTGGG AGCTTGATCC   7380

CCAGGAGGAG GAGGGGTGGG GGGTCCCTGA GTCCCGCCAG GAGAGAGTGG TCGCATACCG   7440

GGAGCCAGTC TGCTGTGGGC CTGTGGGTGG CTGGGGACGG GGGCCAGACA CACAGGCCGG   7500

GAGACGGGTG GGCTGCAGAA CTGTGACTGG TGTGACCGTC GCGATGGGGC CGGTGGTCAC   7560

TGAATCTAAC AGCCTTTGTT ACCGGGGAGT TTCAATTATT TCCCAAAATA AGAACTCAGG   7620

TACAAAGCCA TCTTTCAACT ATCACATCCT GAAAACAAAT GGCAGGTGAC ATTTTCTGTG   7680

CCGTAGCAGT CCCACTGGGC ATTTTCAGGG CCCCTGTGCC AGGGGGGCGC GGGCATCGGC   7740
```

GAGTGGAGGC TCCTGGCTGT GTCAGCCGGC CCAGGGGGAG GAAGGGACCC GGACAGCCAG   7800

AGGTGGGGGG CAGGCTTTCC CCCTGTGACC TGCAGACCCA CTGCACTGCC CTGGCAGGAA   7860

GGGAGGGGAA CTAGGCCAAG GGGGAAGGGC AGGTGCTCTG GAGGGCAAGG GCAGACCTGC   7920

AGACCACCCT GGGGAGCAGG GACTGACCCC CGTCCCTGCC CCATAGTCAG GACCCCGGAG   7980

GTGGACAACG AGGCCCTGGA GAAATTCGAC AAAGCCCTCA AGGCCCTGCC CATGCACATC   8040

CGGCTTGCCT TCAACCCGAC CCAGCTGGAG GGTGAGCACC CAGGCCCCGC CCTTCCCCAG   8100

GGCAGGAGCC ACCCGGCCCC GGGACGACCT CCTCCCATGG TGACCCCCAG CTCCCCAGGC   8160

CTCCCAGGAG GAAGGGGTGG GGTGCAGCAC CCCGTGGGGG CCCCCTCCCC ACCCCCTGCC   8220

AGGCCTCTCT TCCCGAGGTG TCCAGTCCCA TCCTGACCCC CCCATGACTC TCCCTCCCCC   8280

ACAGGGCAGT GCCACGTCTA GGTGAGCCCC TGCCGGTGCC TCTGGGGTAA GCTGCCTGCC   8340

CTGCCCCACG TCCTGGGCAC ACACATGGGG TAGGGGGTCT TGGTGGGGCC TGGGACCCCA   8400

CATCAGGCCC TGGGGTCCCC CCTGTGAGAA TGGCTGGAAG CTGGGGTCCC TCCTGGCGAC   8460

TGCAGAGCTG GCTGGCCGCG TGCCACTCTT GTGGGTGACC TGTGTCCTGG CCTCACACAC   8520

TGACCTCCTC CAGCTCCTTC CAGCAGAGCT AAGGCTAAGT GAGCCAGAAT GGTACCTAAG   8580

GGGAGGCTAG CGGTCCTTCT CCCGAGGAGG GGCTGTCCTG GAACCACCAG CCATGGAGAG   8640

GCTGGCAAGG GTCTGGCAGG TGCCCCAGGA ATCACAGGGG GGCCCCATGT CCATTCAGG   8700

GCCCGGGAGC CTTGGACTCC TCTGGGGACA GACGACGTCA CCACCGCCCC CCCCCCATCA   8760

GGGGGACTAG AAGGGACCAG GACTGCAGTC ACCCTTCCTG GGACCCAGGC CCCTCCAGGC   8820

CCCTCCTGGG GCTCCTGCTC TGGGCAGCTT CTCCTTCACC AATAAAGGCA TAAACCTGTG   8880

CTCTCCCTTC TGAGTCTTTG CTGGACGACG GGCAGGGGGT GGAGAAGTGG TGGGGAGGGA   8940

GTCTGGCTCA GAGGATGACA GCGGGGCTGG GATCCAGGGC GTCTGCATCA CAGTCTTGTG   9000

ACAACTGGGG GCCCACACAC ATCACTGCGG CTCTTTGAAA CTTTCAGGAA CCAGGCAGGG   9060

ACTCGGCAGA GACATCTGCC AGTTCACTTG GAGTGTTCAG TCAACACCCA AACTCCACAA   9120

AGGACAGAAA GTGGAAAATG GCTGTCTCTT AGTCTAATAA ATATTGATAT GAAACTCAAG   9180

TTGCTCATGG ATCAATATGC CTTTATGATC CAGCCAGCCA CTACTGTCGT ATCAACTCAT   9240

GTACCCAAAC GCACTGATCT GTCTGGCTAA TGATGAGAGA TTCCCAGTAG AGAGCTGGCA   9300

AGAGGTCACA GTGAGAACTG TCTGCACACA CAGCAGAGTC CACCAGTCAT CCTAAGGAGA   9360

TCAGTCCTGG TGTTCATTGG AGGACTGATG TTGAAGCTGA AACTCCAATG CTTTGGCCAC   9420

CTGATGTGAA GAGCTGACTC ATTTGAAAAG ACCCTGATGC TGGGAAAGAT TGAGGGCAGG   9480

AGGAGAAGGG GACGACAGAG GATGAGATGG TTGGATGGCA TCACCAACAC AATGGACATG   9540

GGTTTGGGTG GACTCCAGGA GTTGGTGATG GACAGGGAGG CCTGGCGTGC TACGGAAGCG   9600

GTTTATGGGG TCACAAAGAC TGAGTGACTG AACTGAGCTG AACTGAATGG AAATGAGGTA   9660

TACAGCAAAG TGGGGATTTT TTAGATAATA AGAATATACA CATAACATAG TGTATACTCA   9720

TATTTTTATG CATACCTGAA TGCTCAGTCA CTCAGTCGTA TCTGACTCTG TGACCTATGG   9780

ACCGTAGCCT TCCAGGTTTC TTCTGTCCAC AGAATTCTCC AAGGCAAGAA TACTGGAGTG   9840

GGTAGCCATT TCCTCCTCCA GGGGATCCTC CCGACCCAGG GATTGAACCG GCATCTCCTG   9900

TATTGGCAGG TGGATTCTTT ACCACTGTGC CACCAGGGAA GCCCGTGTTA CTCTCTATGT   9960

CCCACTTAAT TACCAAAGCT GCTCCAAGAA AAAGCCCCTG TGCCCTCTGA GCTTCCCGGC   10020

CTGCAGAGGG TGGTGGGGGT AGACTGTGAC CTGGGAACAC CCTCCCGCTT CAGGACTCCC   10080

GGGCCACGTG ACCCACAGTC CTGCAGACAG CCGGGTAGCT CTGCTCTTCA AGGCTCATTA   10140

TCTTTAAAAA AAACTGAGGT CTATTTTGTG ACTTCGCTGC CGTAACTTCT GAACATCCAG   10200

TGCGATGGAC AGGACCTCCT CCCCAGGCCT CAGGGGCTTC AGGGAGCCAG CCTTCACCTA   10260

TGAGTCACCA GACACTCGGG GGTGGCCCCG CCTTCAGGGT GCTCACAGTC TTCCCATCGT   10320

CCTGATCAAA GAGCAAGACC AATGACTTCT TAGGAGCAAG CAGACACCCA CAGGACACTG   10380

AGGTTCACCA GAGCTGAGCT GTCCTTTTGA ACCTAAAGAC ACACAGCTCT CGAAGGTTTT   10440

CTCTTTAATC TGGATTTAAG GCCTACTTGC CCCTCAAGAG GGAAGACAGT CCTGCATGTC   10500

CCCAGGACAG CCACTCGGTG GCATCCGAGG CCACTTAGTA TTATCTGACC GCACCCTGGA   10560

ATTAATCGGT CCAAACTGGA CAAAAACCTT GGTGGGAAGT TTCATCCCAG AGGCCTCAAC   10620

CATCCTGCTT TGACCACCCT GCATCTTTTT TTCTTTTATG TGTATGCATG TATA¨ATATA   10680

TATATATTTT TTTTTTTTTC ATTTTTTGGC TGTGCTGGCT GTTCGTTGCA GTTCGGTGCG   10740

CAGGCTTCTC TCTAGTTTCT CTCTAGTCTT CTCTTATCAC AGAGCAGTCT CTAGACGATC   10800

GACGCGT

                                                                   10807

(2) INFORMATION FOR SEQ ID NO:6:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 47 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AATTCCGATC GACGCGTCGA CGATATACTC TAGACGATCG ACGCGTA   47

(2) INFORMATION FOR SEQ ID NO:7:

        (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 47 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

AAGCTACGCG TCGATCGTCT AGAGTATATC GTCGACGCGT CGATCGG   47

(2) INFORMATION FOR SEQ ID NO:8:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGGATCCCCT GCCGGTGCCT CTGG   24

(2) INFORMATION FOR SEQ ID NO:9:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 24 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AACGCGTCAT CCTCTGTGAG CCAG   24

(2) INFORMATION FOR SEQ ID NO:10:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 10 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6839

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

ACTACGTAGT   10

(2) INFORMATION FOR SEQ ID NO:11:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC962

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGTCACCTGA GAAGAAAACG AGACA   25

(2) INFORMATION FOR SEQ ID NO:12:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6303

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

ATTTGCGGCC GCCTGCAGCC ATGTGGCAGC TCACAAGCCT CCTGC   45

(2) INFORMATION FOR SEQ ID NO:13:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 45 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6337

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CAGGAAGGAG TTGGCGCGCT TGCGCCGTTG CAGCACCTGG TGGGC   45

(2) INFORMATION FOR SEQ ID NO:14:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 25 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6306

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTTCTTCCTG AATTCTGTTT CTTGC   25

(2) INFORMATION FOR SEQ ID NO:15:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6338

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

CGGATCCGCA AGCGCGCCAA CTCCTTCC   28

(2) INFORMATION FOR SEQ ID NO:16:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 28 base pairs
        (B) TYPE: nucleic acid

            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (vii) IMMEDIATE SOURCE:
            (B) CLONE: ZC6373

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

AAAGTAAAAA AAGATCTAAA AATTTAAC   28

(2) INFORMATION FOR SEQ ID NO:17:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 32 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (vii) IMMEDIATE SOURCE:
            (B) CLONE: ZC6305

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

GTGTCTCGTT TTCTTCTTAA GTGACTGCGC TT   32

(2) INFORMATION FOR SEQ ID NO:18:

      (i) SEQUENCE CHARACTERISTICS:
            (A) LENGTH: 49 base pairs
            (B) TYPE: nucleic acid
            (C) STRANDEDNESS: single
            (D) TOPOLOGY: linear

      (vii) IMMEDIATE SOURCE:
            (B) CLONE: ZC6302

      (xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

TTAAGAAGAA AACGAGACAC AGAAGACCAA GAAGACCAAG TAGATCCGC   49

(2) INFORMATION FOR SEQ ID NO:19:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 43 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (vii) IMMEDIATE SOURCE:
        (B) CLONE: ZC6304

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

GGATCTACTT GGTCTTCTTG GTCTTCTGTG TCTCGTTTTC TTC   43

(2) INFORMATION FOR SEQ ID NO:20:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

    Arg Arg Lys Arg
    1

(2) INFORMATION FOR SEQ ID NO:21:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 4 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

    Lys Arg Lys Arg
    1

(2) INFORMATION FOR SEQ ID NO:22:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 8 amino acids
        (B) TYPE: amino acid
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

Ser His Leu Arg Arg Lys Arg Asp
1   5

(2) INFORMATION FOR SEQ ID NO:23:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 6763 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
ACGCGTCGAC CTGCAGGTCA ACGGATCTCT GTGTCTGTTT TCATGTTAGT ACCACACTGT      60

TTTGGTGGCT GTAGCTTTCA GCTACAGTCT GAAGTCATAA AGCCTGGTAC CTCCAGCTCT     120

GTTCTCTCTC AAGATTGTGT TCTGCTGTTT GGGTCTTTAG TGTCTCCACA CAATTTTAG     180

AATTGTTTGT TCTAGTTCTG TGAAAAATGA TGCTGGTATT TTGATAAGGA TTGCATGAA     240

TCTGTAAAGC TACAGATATA GTCATTGGGT AGTACAGTCA CTTTAACAAT ATTAACTCTT     300

CACATCTGTG AGCATGATAT ATTTTCCCCC TCTATATCAT CTTCAATTCC TCCTACAGT     360

TTCTTTCATT GCAGTTTTCT GAGTACAGGT CTTACACCTC CTTGGTTAGA GTCATCCTC     420

AGTATTTTAT TCCTTTGATA CAATTGTGAA TGAGGTAATT TTCTTAGTTT CTCTTCTGA     480

TAGCTCATTG TTAGTGTATA TATAGAAAAG CAACAGATTT CTATGTATTA ATTTTGTATC     540
```

```
CTGCAACAGA TTTCTATGTA TTAATTTTGT ATCCTGCTAC TTTACGGAAT TCACTTATTA    600

GCTTTTTGGT GACATCTTGA GGATTTTCTG AAGAAAATGG CATGGTATGG TAGGACAAGG    660

TGTCATGTCA TCTGCAAACA GTGGCAGTTT TCCTTCTTCC CTTCCAACCT GGATTTCTTT    720

GATTTCTTTC TGTCTGAGTA CGACTAGGAT TCCCAATACT ATACCGAATA AAAGTGGCAA    780

GAGTGGACAT CCTTGTCTTA TTTTTCTGAC CTTAGAGGAA ATGCTTTCAG TTTTTCACCA    840

TTAATTATAA TGTTTACTGT GGGCTTGTCA TATGTGGCCT TCATTATATG GAGGTCTATT    900

CCCTCTATAC CCACCTTGTT GAGAGTTTTT ATCATAAAAG TATGTTGAAT TTTGTCAAAA    960

GTTTTTCCTG CATCTATTGA GATGATTTTT ACTCTTCAAT TCATTAATGA TTTTTATTCT   1020

TCATTTTGTT AATGATTTCC ATTCTTCAAT TTGTTAACGT GGTATATCAC ATTGATTGAT   1080

TTGTGGATAC CTTTGTATCC CTGGGATAAA CCTCACTTGA TCATGAGCTT TCAATGTATT   1140

TTTGAATTCA CTTTGCTAAT ATTCTGTTGG GTATTTTTGC ATCTCTATTC ATCAATGATA   1200

TTGGCCTAAG AAAGGTTTTG TCTGGTTTTA GTATCAGGGT GATGCTGGCC TCATAGAGAG   1260

AGTTTAGAAG CATTTCCTCC TCTTTGATTT TTCGGAATAG TTTGAGTAGG ATAGGTATTA   1320

ACTCTTCTTT AAATGTTTGG GGACTTCCCT GGTGAGCCGG TGGTTGAGAA TCCGCCTCAG   1380

GGATGTGGGT TTGATCCCTG GTCAGGGAAC CATTAATAAG ATCCCACATG CTGCAGGCAA   1440

CAAGCCCCCA AGCTGCAACC ACTGAGCTGC AACCGCTGCA GTGCCCACAG GCCACGACCA   1500

GAGAAAGCCC ACATACAGCA GGGAAGACCC AGCACAACCG GAAAAAGGAG TTTGGTGGAA   1560

TACAGCTGTG AAGCCGTCTG GTCCTGGACT CCTGCTTGAG GGAATTTTTT AAAAATTATT   1620

GATTCAATTT CATTACTGGT AACTGGTCTG TTCATATTTT CTATTTCTTC CGGGTTCAGT   1680

CTTGGGAGAT TGTACATGCC TAGGAATGTG TCCGTTTCTT CTAGGTTGTC CATTTTATTG   1740

GACATGCATG GGAGCACACA GCACCGACCA GCGAGACTCA TGCTGGCTTC CTGGGGCCAG   1800

GCTGGGGCCC CAAGCAGCAT GGCATCCTAG AGTGTGTGAA AGCCCACTGA CCCTGCCCAG   1860

CCCCACAATT TCATTCTGAG AAGTGATTCC TTGCTTCTGC ACTTACAGGC CCAGGATCTG   1920
```

```
ACCTGCTTCT GAGGAGCAGG GGTTTTGGCA GGACGGGGAG ATGCTGAGAG CCGACGGGGG    1980

TCCAGGTCCC CTCCCAGGCC CCCCTGTCTG GGGCAGCCCT TGGGAAAGAT TGCCCCAGTC    2040

TCCCTCCTAC AGTGGTCAGT CCCAGCTGCC CCAGGCCAGA GCTGCTTTAT TTCCGTCTCT    2100

CTCTCTGGAT GGTATTCTCT GGAAGCTGAA GGTTCCTGAA GTTATGAATA GCTTTGCCCT    2160

GAAGGGCATG GTTTGTGGTC ACGGTTCACA GGAACTTGGG AGACCCTGCA GCTCAGACGT    2220

CCCGAGATTG GTGGCACCCA GATTTCCTAA GCTCGCTGGG AACAGGGCG CTTGTTCTC     2280

CCTGGCTGAC CTCCCTCCTC CCTGCATCAC CCAGTTCTGA AAGCAGAGCG GTGCTGGGGT    2340

CACAGCCTCT CGCATCTAAC GCCGGTGTCC AAACCACCCG TGCTGGTGTT CGGGGGGCTA    2400

CCTATGGGGA AGGGCTTCTC ACTGCAGTGG TGCCCCCCGT CCCCTCTGAG ATCACAAGTC    2460

CCAGTCCGGA CGTCAAACAG GCCGAGCTCC CTCCAGAGGC TCCAGGGAGG GATCCTTGCC    2520

CCCCCGCTGC TGCCTCCAGC TCCTGGTGCC GCACCCTTGA GCCTGATCTT GTAGACGCCT    2580

CAGTCTAGTC TCTGCCTCCG TGTTCACACG CCTTCTCCCC ATGTCCCCTC CGTGTCCCCG    2640

TTTTCTCTCA CAAGGACACC GGACATTAGA TTAGCCCCTG TTCCAGCCTC ACCTGAACAG    2700

CTCACATCTG TAAAGACCTA GATTCCAAAC AAGATTCCAA CCTGAAGTTC CCGGTGGATG    2760

TGAGTTCTGG GGCGACATCC TTCAACCCCA TCACAGCTTG CAGTTCATCG CAAAACATGG    2820

AACCTGGGGT TTATCGTAAA ACCCAGGTTC TTCATGAAAC ACTGAGCTTC GAGGCTTGTT    2880

GCAAGAATTA AAGGTGCTAA TACAGATCAG GGCAAGGACT GAAGCTGGCT AAGCCTCCTC    2940

TTTCCATCAC AGGAAAGGGG GGCCTGGGGG CGGCTGGAGG TCTGCTCCCG TGAGTGAGCT    3000

CTTTCCTGCT ACAGTCACCA ACAGTCTCTC TGGGAAGGAA ACCAGAGGCC AGAGAGCAAG    3060

CCGGAGCTAG TTTAGGAGAC CCCTGAACCT CCACCCAAGA TGCTGACCAG CCAGCGGGCC    3120

CCCTGGAAAG ACCCTACAGT TCAGGGGGGA AGAGGGGCTG ACCCGCCAGG TCCCTGCTAT    3180

CAGGAGACAT CCCCGCTATC AGGAGATTCC CCCACCTTGC TCCCGTTCCC CTATCCCAAT    3240
```

```
ACGCCCACCC CACCCCTGTG ATGAGCAGTT TAGTCACTTA GAATGTCAAC TGAAGGCTTT    3300

TGCATCCCCT TTGCCAGAGG CACAAGGCAC CCACAGCCTG CTGGGTACCG ACGCCCATGT    3360

GGATTCAGCC AGGAGGCCTG TCCTGCACCC TCCCTGCTCG GCCCCCTCT GTGCTCAGCA     3420

ACACACCCAG CACCAGCATT CCCGCTGCTC CTGAGGTCTG CAGGCAGCTC GCTGTAGCCT    3480

GAGCGGTGTG GAGGGAAGTG TCCTGGGAGA TTTAAAATGT GAGAGGCGGG AGGTGGGAGG    3540

TTGGGCCCTG TGGGCCTGCC CATCCCACGT GCCTGCATTA GCCCCAGTGC TGCTCAGCCG    3600

TGCCCCCGCC GCAGGGGTCA GGTCACTTTC CCGTCCTGGG GTTATTATGA CTCTTGTCAT    3660

TGCCATTGCC ATTTTTGCTA CCCTAACTGG GCAGCAGGTG CTTGCAGAGC CCTCGATACC    3720

GACCAGGTCC TCCCTCGGAG CTCGACCTGA ACCCCATGTC ACCCTTGCCC CAGCCTGCAG    3780

AGGGTGGGTG ACTGCAGAGA TCCCTTCACC CAAGGCCACG GTCACATGGT TTGGAGGAGC    3840

TGGTGCCCAA GGCAGAGGCC ACCCTCCAGG ACACACCTGT CCCCAGTGCT GGCTCTGACC    3900

TGTCCTTGTC TAAGAGGCTG ACCCCGGAAG TGTTCCTGGC ACTGGCAGCC AGCCTGGACC    3960

CAGAGTCCAG ACACCCACCT GTGCCCCCGC TTCTGGGGTC TACCAGGAAC CGTCTAGGCC    4020

CAGAGGGGAC TTCCTGCTTG GCCTTGGATG GAAGAAGGCC TCCTATTGTC CTCGTAGAGG    4080

AAGCCACCCC GGGGCCTGAG GATGAGCCAA GTGGGATTCC GGGAACCGCG TGGCTGGGGG    4140

CCCAGCCCGG GCTGGCTGGC CTGCATGCCT CCTGTATAAG GCCCCAAGCC TGCTGTCTCA    4200

GCCCTCCACT CCCTGCAGAG CTCAGAAGCA CGACCCCAGG GATATCATCG ATAAGCTTGG    4260

ATCCCCTGCC GGTGCCTCTG GGGTAAGCTG CCTGCCCTGC CCCACGTCCT GGGCACACAC    4320

ATGGGGTAGG GGGTCTTGGT GGGGCCTGGG ACCCCACATC AGGCCCTGGG GTCCCCCCTG    4380

TGAGAATGGC TGGAAGCTGG GGTCCCTCCT GGCGACTGCA GAGCTGGCTG GCCGCGTGCC    4440

ACTCTTGTGG GTGACCTGTG TCCTGGCCTC ACACACTGAC CTCCTCCAGC TCCTTCCAGC    4500

AGAGCTAAGG CTAAGTGAGC CAGAATGGTA CCTAAGGGGA GGCTAGCGGT CCTTCTCCCG    4560

AGGAGGGGCT GTCCTGGAAC CACCAGCCAT GGAGAGGCTG GCAAGGGTCT GGCAGGTGCC    4620
```

```
CCAGGAATCA CAGGGGGGCC CCATGTCCAT TTCAGGGCCC GGGAGCCTTG GACTCCTCTG   4680

GGGACAGACG ACGTCACCAC CGCCCCCCCC CCATCAGGGG GACTAGAAGG GACCAGGACT   4740

GCAGTCACCC TTCCTGGGAC CCAGGCCCCT CCAGGCCCCT CCTGGGGCTC CTGCTCTGGG   4800

CAGCTTCTCC TTCACCAATA AAGGCATAAA CCTGTGCTCT CCCTTCTGAG TCTTTGCTGG   4860

ACGACGGGCA GGGGGTGGAG AAGTGGTGGG GAGGGAGTCT GGCTCAGAGG ATGACAGCGG   4920

GGCTGGGATC CAGGGCGTCT GCATCACAGT CTTGTGACAA CTGGGGGCCC ACACACATCA   4980

CTGCGGCTCT TTGAAACTTT CAGGAACCAG GGAGGGACTC GGCAGAGACA TCTGCCAGTT   5040

CACTTGGAGT GTTCAGTCAA CACCCAAACT CGACAAAGGA CAGAAAGTGG AAAATGGCTG   5100

TCTCTTAGTC TAATAAATAT TGATATGAAA CTCAAGTTGC TCATGGATCA ATATGCCTTT   5160

ATGATCCAGC CAGCCACTAC TGTCGTATCA ACTCATGTAC CCAAACGCAC TGATCTGTCT   5220

GGCTAATGAT GAGAGATTCC CAGTAGAGAG CTGGCAAGAG GTCACAGTGA GAACTGTCTG   5280

CACACACAGC AGAGTCCACC AGTCATCCTA AGGAGATCAG TCCTGGTGTT CATTGGAGGA   5340

CTGATGTTGA AGCTGAAACT CCAATGCTTT GGCCACCTGA TGTGAAGAGC TGACTCATTT   5400

GAAAAGACCC TGATGCTGGG AAAGATTGAG GGCAGGAGGA GAAGGGGACG ACAGAGGATG   5460

AGATGGTTGG ATGGCATCAC CAACACAATG GACATGGGTT TGGGTGGACT CCAGGAGTTG   5520

GTGATGGACA GGGAGGCCTG GCGTGCTACG GAAGCGGTTT ATGGGGTCAC AAAGACTGAG   5580

TGACTGAACT GAGCTGAACT GAATGGAAAT GAGGTATACA GCAAAGTGGG GATTTTTTAG   5640

ATAATAAGAA TATACACATA ACATAGTGTA TACTCATATT TTTATGCATA CCTGAATGCT   5700

CAGTCACTCA GTCGTATCTG ACTCTGTGAC CTATGGACCG TAGCCTTCCA GGTTTCTTCT   5760

GTCCACAGAA TTCTCCAAGG CAAGAATACT GGAGTGGGTA GCCATTTCCT CCTCCAGGGG   5820

ATCCTCCCGA CCCAGGGATT GAACCGGCAT CTCCTGTATT GGCAGGTGGA TTCTTTACCA   5880

CTGTGCCACC AGGGAAGCCC GTGTTACTCT CTATGTCCCA CTTAATTACC AAAGCTGCTC   5940
```

```
CAAGAAAAAG CCCCTGTGCC CTCTGAGCTT CCCGGCCTGC AGAGGGTGGT GGGGGGTAGAC    6000

TGTGACCTGG GAACACCCTC CCGCTTCAGG ACTCCCGGGC CACGTGACCC ACAGTCCTGC    6060

AGACAGCCGG GTAGCTCTGC TCTTCAAGGC TCATTATCTT TAAAAAAAAC TGAGGTCTAT    6120

TTTGTGACTT CGCTGCCGTA ACTTCTGAAC ATCCAGTGCG ATGGACAGGA CCTCCTCCCC    6180

AGGCCTCAGG GGCTTCAGGG AGCCAGCCTT CACCTATGAG TCACCAGACA CTCGGGGGTG    6240

GCCCCGCCTT CAGGGTGCTC ACAGTCTTCC CATCGTCCTG ATCAAAGAGC AAGACCAATG    6300

ACTTCTTAGG AGCAAGCAGA CACCCACAGG ACACTGAGGT TCACCAGAGC TGAGCTGTCC    6360

TTTTGAACCT AAAGACACAC AGCTCTCGAA GGTTTTCTCT TTAATCTGGA TTTAAGGCCT    6420

ACTTGCCCCT CAAGAGGGAA GACAGTCCTG CATGTCCCCA GGACAGCCAC TCGGTGGCAT    6480

CCGAGGCCAC TTAGTATTAT CTGACCGCAC CCTGGAATTA ATCGGTCCAA ACTGGACAAA    6540

AACCTTGGTG GGAAGTTTCA TCCCAGAGGC CTCAACCATC CTGCTTTGAC CACCCTGCAT    6600

CTTTTTTTCT TTTATGTGTA TGCATGTATA TATATATATA TATTTTTTTT TTTTTCATTT    6660

TTTGGCTGTG CTGGCTGTTC GTTGCAGTTC GGTGCGCAGG CTTCTCTCTA GTTTCTCTCT    6720

AGTCTTCTCT TATCACAGAG CAGTCTCTAG ACGATCGACG CGT                      6763
```

(2) INFORMATION FOR SEQ ID NO:24:

    (i) SEQUENCE CHARACTERISTICS:
       (A) LENGTH: 5 amino acids
       (B) TYPE: amino acid
       (D) TOPOLOGY: linear

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

    Arg Ile Arg Lys Arg
    1   5

(2) INFORMATION FOR SEQ ID NO:25:

    (i) SEQUENCE CHARACTERISTICS:

    (A) LENGTH: 5 amino acids
    (B) TYPE: amino acid
    (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

Gln Arg Arg Lys Arg
1   5

CLAIMS

1. A method for producing protein C in a transgenic animal comprising:

providing a DNA construct comprising a first DNA segment encoding a secretion signal and a protein C propeptide operably linked to a second DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lysine (Lys)-Arginine (Arg) to R_1-R_2-R_3-R_4, and wherein each of R_1, R_2, R_3, R_4 is individually Lys or Arg, and wherein said first and second segments are operably linked to additional DNA segments required for expression of the protein C DNA in a mammary gland of a host female animal;

introducing said DNA construct into a fertilized egg of a non-human mammalian species;

inserting said egg into an oviduct or uterus of a female of said species to obtain offspring carrying said DNA construct;

breeding said offspring to produce female progeny that express said first and second DNA segments and produce milk containing protein C encoded by said second segment, wherein said protein has anticoagulant activity upon activation;

collecting milk from said female progeny; and

recovering the protein C from the milk.

2. The method of claim 1, further comprising the step of activating the protein C.

3. The method of claim 1, wherein R_1-R_2-R_3-R_4 is Arg-Arg-Lys-Arg (SEQ ID NO: 20).

4. The method of claim 1, wherein said species is selected from sheep, rabbits, cattle and goats.

5.   The method of claim 1, wherein each of said first and second DNA segments comprises an intron.

6.   The method of claim 1, wherein the second DNA segment comprises a DNA sequence of nucleotides as shown in Seq. ID NO: 1 or Seq. ID. NO: 3.

7.   The method of claim 6, wherein the second DNA segment comprises the DNA sequence of nucleotides as shown in SEQ. ID. NO: 1.

8.   The method of claim 1, wherein the additional DNA segments comprise a transcriptional promoter selected from the group consisting of casein, β-lactoglobulin, α-lactalbumin and whey acidic protein gene promoters.

9.   The method of claim 8, wherein the transcriptional promoter is the β-lactoglobulin gene promoter.

10.   A transgenic non-human female mammal that produces recoverable amounts of human protein C in its milk, wherein at least 90% of the human protein C in the milk is two-chain protein C.

11.   A process for producing a transgenic offspring of a mammal comprising:

providing a DNA construct comprising a first DNA segment encoding a secretion signal and a protein C propeptide operably linked to a second DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of R_1, R_2, R_3, R_4, is individually Lys or Arg, and wherein said first and second segments are operably linked to additional DNA segments required for expression of the protein C DNA in the mammary gland of a host female animal;

introducing said DNA construct into a fertilized egg of a non-human mammalian species; and

inserting said egg into an oviduct or uterus of a female of said species to obtain offspring carrying said DNA construct.

12. The process according to claim 11, wherein R_1-R_2-R_3-R_4 is Arg-Arg-Lys-Arg (SEQ ID NO: 20).

13. The process according to claim 11, wherein the offspring is female.

14. The process according to claim 11, wherein the offspring is male.

15. A non-human mammal produced according to the process of claim 10.

16. A non-human mammal of claim 15, wherein the mammal is female.

17. A female mammal according to claim 16 that produces milk containing protein C encoded by said DNA construct, wherein said protein C has anticoagulant activity upon activation.

18. A non-human mammalian embryo containing in its nucleus a heterologous DNA segment encoding protein C, wherein the encoded protein C comprises a two-chain cleavage site modified from Lys-Arg to R_1-R_2-R_3-R_4, and wherein each of R_1, R_2, R_3, R_4, is individually Lys or Arg.

← Single Chain

← Heavy Chain

← Light Chain

1   2
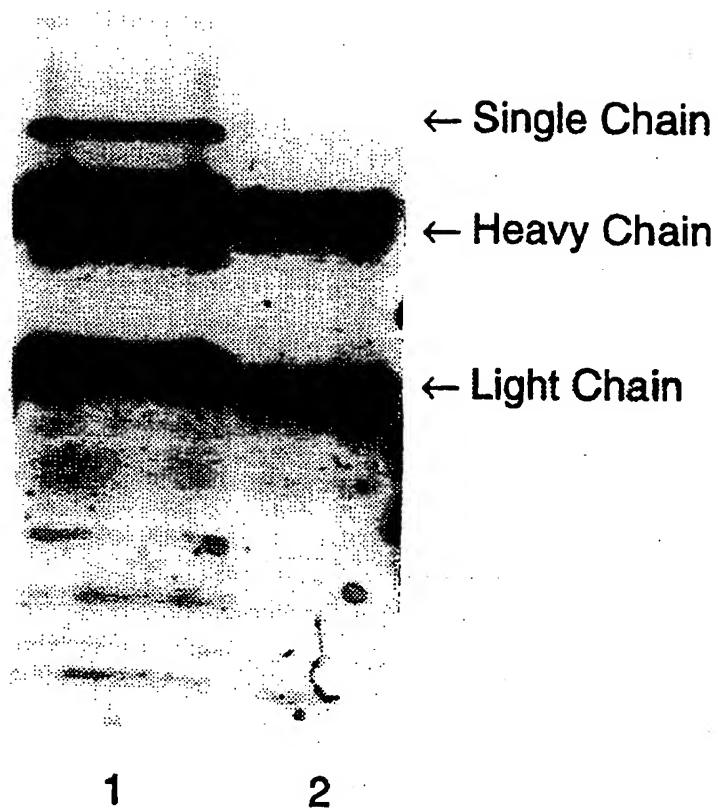
Fig. 1
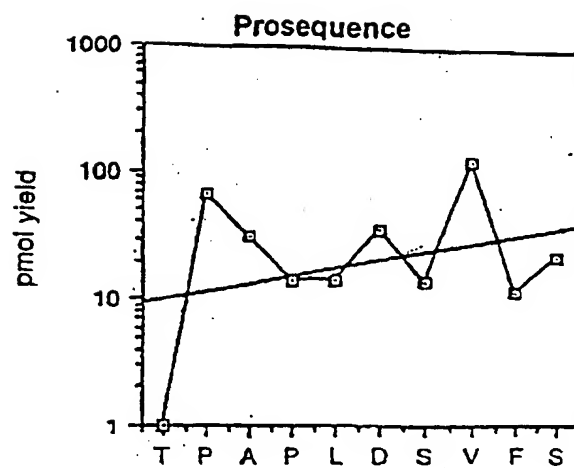
Fig. 2a
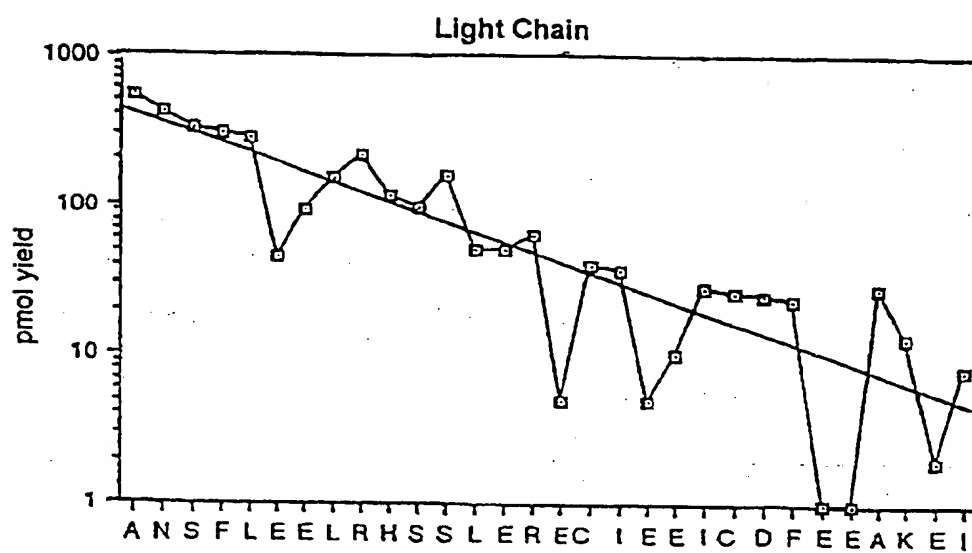
Fig. 2b
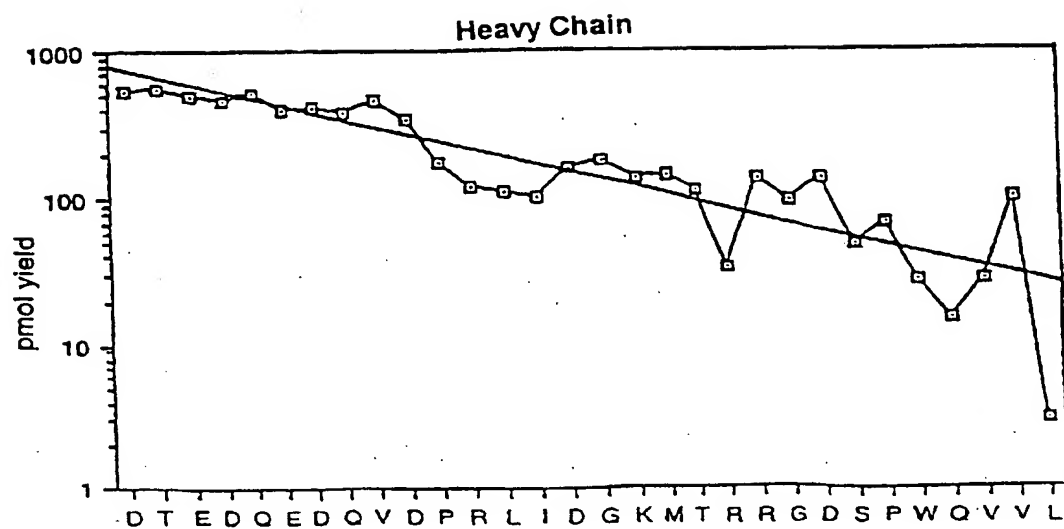
Fig. 2c
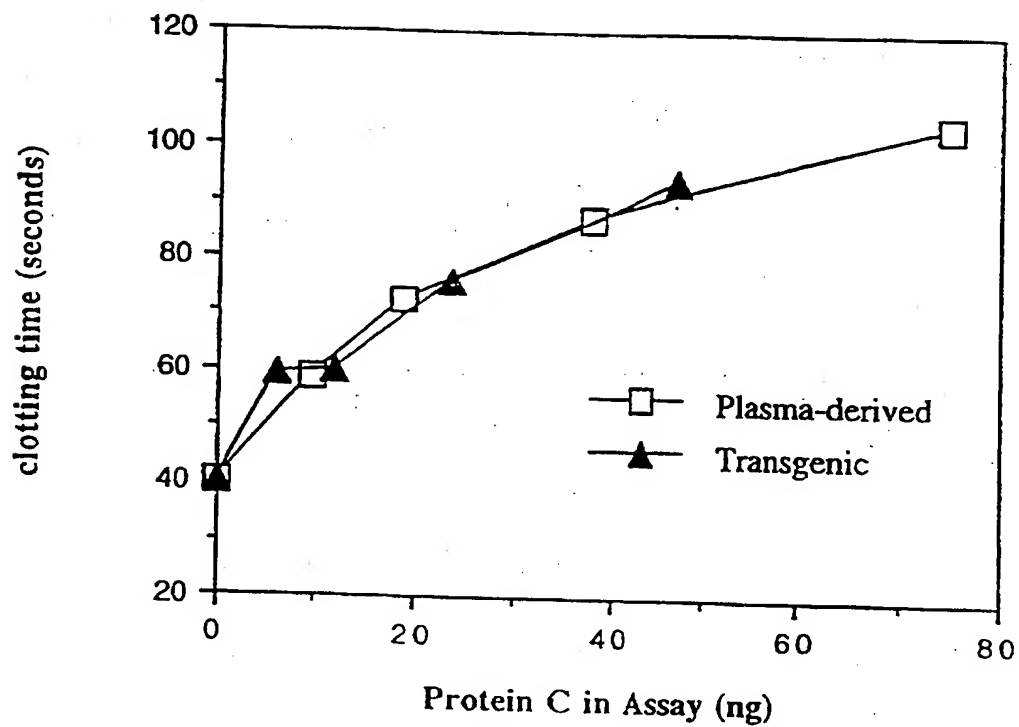
Fig. 3

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6   C12N15/00   C12N9/64   A01K67/027

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6   C12N   A01K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TRANSGENIC RESEARCH, vol. 3, 1994, pages 355-364, XP000647718 W. DROHAN ET AL: "Inefficient processing of human protein C in the mouse mammary gland" cited in the application see the whole document especially page 362, right column, lines 11-19 --- -/--	1-18

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
25 March 1997	0 2. 04. 97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Van der Schaal, C

2

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	MIAMI BIO/TECHNOLOGY WINTER SYMPOSIUM ON ADVANCES IN GENE TECHNOLOGY: PROTEIN ENGINEERING AND STRUCTURAL BIOLOGY, MIAMI, FLORIDA, USA, FEBRUARY 4-9, 1995. PROTEIN ENGINEERING 8 (SUPPL.). 1995. 107. ISSN: 0269-2139, XP002028254 COLMAN A ET AL: "The transgenic mammary gland as a bioreactor: Expectations and realisations." see page 107, left-hand column, paragraph 5 last lines	1-18
Y	BIOCHEMISTRY, vol. 29, 1990, pages 347-354, XP002028255 D. FOSTER ET AL: "Endoproteolytic processing of the dibasic cleavage site in the human protein C precursor in transfected mammalia cells: Effects of sequence alterations on efficiency of cleavage" see the whole document	1-18
Y	EP 0 319 312 A (LILLY CO ELI) 7 June 1989 see the whole document	1-18
Y	WO 88 00239 A (PHARMACEUTICAL PROTEINS LTD) 14 January 1988 cited in the application see the whole document	4,8,9
Y	WO 92 11757 A (AMERICAN NAT RED CROSS) 23 July 1992 see the whole document especially page 11 and 12	1-18
P,A	WO 96 34966 A (AMERICAN NAT RED CROSS) 7 November 1996	

2

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0319312 A	07-06-89	US 4992373 A	12-02-91
		AU 2650988 A	08-06-89
		CN 1035527 A	13-09-89
		DE 3885419 D	09-12-93
		DE 3885419 T	31-03-94
		ES 2059538 T	16-11-94
		HU 209587 B	29-08-94
		IE 61627 B	16-11-94
		IL 88559 A	27-02-94
		JP 2002372 A	08-01-90
		PT 89135 B	29-04-94
		SU 1830081 A	23-07-93
WO 8800239 A	14-01-88	AU 605497 B	17-01-91
		AU 7649087 A	29-01-88
		EP 0274489 A	20-07-88
		JP 1500162 T	26-01-89
		US 5366894 A	22-11-94
		US 5322775 A	21-06-94
		US 5476995 A	19-12-95
WO 9211757 A	23-07-92	AU 1228592 A	17-08-92
		EP 0591219 A	13-04-94
		JP 6507307 T	25-08-94
		US 5589604 A	31-12-96
WO 9634966 A	07-11-96	AU 6347496 A	21-11-96

THIS PAGE BLANK (USPTO)

(51) International Patent Classification ⁶ :		(11) International Publication Number:	**WO 98/48818**
A61K 31/725	**A1**	(43) International Publication Date:	5 November 1998 (05.11.98)

(21) International Application Number: PCT/US98/08386

(22) International Filing Date: 24 April 1998 (24.04.98)

(30) Priority Data:
60/045,255 28 April 1997 (28.04.97) US

(71) Applicant *(for all designated States except US)*: ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: CARLSON, Andrew, David [US/US]; 8020 Castle Cove, Indianapolis, IN 46256 (US). SHELIGA, Theodore, Arsay [US/US]; 7901 Venetian Way, Indianapolis, IN 46217 (US).

(74) Agents: CALTRIDER, Steven, P. et al.; Eli Lilly and Company, Lilly Corporate Center, Indianapolis, IN 46285 (US).

(81) Designated States: AL, AM, AU, AZ, BA, BB, BG, BR, BY, CA, CN, CU, CZ, EE, GE, GH, GM, GW, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, RO, RU, SD, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ACTIVATED PROTEIN C FORMULATIONS

(57) Abstract

The present invention relates to pharmaceutical formulations of activated protein C which also comprises sucrose, sodium chloride and sodium citrate buffer at a pH between about 5.5 and about 6.5. The activated protein C formulations of the present invention are more stable than other formulations of activated protein C and demonstrate fewer degradation products over time.

Title

ACTIVATED PROTEIN C FORMULATIONS

This application claims the benefit of U.S. Provisional
5 Application No. 60/045,255, filed April 28, 1997.

Field of the Invention

This invention is in the field of human medicine,
particularly in the treatment of vascular disorders with
10 activated protein C. More specifically, the present
invention relates to formulations of activated human
protein C.

Background of the Invention

15 Protein C is a serine protease and naturally occurring
anticoagulant that plays a role in the regulation of
homeostasis by inactivating Factors Va and VIIIa in the
coagulation cascade. Human protein C is made *in vivo*
primarily in the liver as a single polypeptide of 461 amino
20 acids. This single chain precursor molecule undergoes
multiple post-translational modifications including 1)
cleavage of a 42 amino acid signal sequence; 2) proteolytic
removal from the one chain zymogen of the lysine residue at
position 156 and the arginine residue at position 157 to
25 make a 2-chain zymogen form of the molecule, (i.e., a light
chain of 155 amino acid residues attached through a
disulfide bridge to the serine protease-containing heavy
chain of 262 amino acid residues); 3) vitamin K-dependent
carboxylation of nine glutamic acid residues clustered in
30 the first 42 amino acids of the light chain, resulting in
nine gamma-carboxyglutamic acid residues; and 4)
carbohydrate attachment at four sites (one in the light

chain and three in the heavy chain). The heavy chain contains the well established serine protease triad of Asp 257, His 211 and Ser 360. Finally, the circulating 2-chain zymogen is activated *in vivo* by thrombin at a phospholipid
5 surface in the presence of calcium ion. Activation results from removal of a dodecapeptide at the N-terminus of the heavy chain, producing activated protein C (aPC) possessing enzymatic activity.

In addition to the enzymatic activities of aPC within
10 the blood coagulation cascade, aPC also can autodegrade, leading to decreased functionality as an anticoagulant. Applicants have discovered an important degradation pathway. Autodegradation of the N-terminus of the light chain may result in a clip on either side of the histidine residue at
15 position 10. Thus, this degradation pathway yields two inactive products: 1) des(1-9) activated protein C, wherein the first nine N-terminal residues of the light chain have been removed; and 2) des(1-10) activated protein C, wherein the first ten N-terminal residues of the light chain have
20 been removed. This degradation pathway, which has not been previously reported, results in loss of anticoagulant activity due to the removal of the critical GLA residues at positions 6 and 7. Therefore, minimizing the level of the des(1-9) and des(1-10) activated Protein C autodegradation
25 products is important in achieving a potent, high purity, activated protein C pharmaceutical formulation. These variants were previously unknown degradation products and are exceedingly difficult, if not impossible, to remove by conventional purification techniques. Applicants have
30 further discovered that solid-state solubility is significantly enhanced in the presence of a select group of bulking agents.

It is clearly desirable to minimize such degradation of activated protein C in both the solution and lyophilized
35 solid states. Accordingly, these discoveries allow the preparation of potent, high purity, activated protein C

formulations which are pharmaceutically elegant to the health care provider.

The present invention provides improved formulations of activated protein C substantially free of such

5   autodegradation products, particularly, des(1-9) and des(1-10) forms of the light chain of activated protein C. Therefore, said formulations are suitable for administration to a patient in need thereof.

Summary of the Invention

10   The present invention provides a stable lyophilized formulation comprising activated protein C and a bulking agent selected from the group consisting of mannitol, trehalose, raffinose, sucrose, and mixtures thereof.

The present invention also provides a stable

15   lyophilized formulation comprising about 2.5 mg/mL activated protein C, about 15 mg/mL sucrose, and about 20 mg/mL NaCl. Furthermore, the present invention provides a stable lyophilized formulation comprising about 5 mg/mL activated protein C, about 30 mg/mL sucrose, and about 38 mg/mL NaCl.

20   The present invention also provides a process for preparing a formulation comprising activated protein C and a bulking agent selected from the group consisting of mannitol, trehalose, raffinose, and sucrose and mixtures

25   thereof.

The invention also provides a unit dosage form comprising a unit dosage receptacle containing the formulation wherein the weight to weight ratio is about 1 part activated protein C, about 7.6 parts salt and about

30   6 parts bulking agent.

The invention further provides a method of treating disease states involving intravascular coagulation comprising the administration of a formulation of activated protein C described herein.

35

Detailed Description of the Invention

For purposes of the present invention, as disclosed and claimed herein, the following terms are as defined below.

aPC or activated protein C refers to activated protein C whether recombinant or plasma derived. aPC includes and is preferably human activated protein C although aPC may also include other species or derivatives having protein C proteolytic, amidolytic, esterolytic, and biological (anticoagulant or pro-fibrinolytic) activities. Examples of protein C derivatives are described by Gerlitz, et al., U.S. patent No. 5,453,373, and Foster, et al., U.S. patent No. 5,516,650, the entire teachings of which are hereby incorporated by reference.

APTT - activated partial thromboplastin time.

r-hPC- recombinant human protein C zymogen.

r-aPC - recombinant activated protein C produced by activating protein C zymogen *in vitro* or *in vivo* or by direct secretion of the activated form of protein C from procaryotic cells, eukaryotic cells, or transgenic animals including, for example, secretion from human kidney 293 cells as a zymogen then purified and activated by techniques well known to the skilled artisan and demonstrated in Yan, U.S. Patent No. 4,981,952, and Cottingham, WO 97/20043, the entire teachings of which are herein incorporated by reference.

Continuous infusion - continuing substantially uninterrupted the introduction of a solution into a blood vessel for a specified period of time.

Bolus injection - the injection of a drug in a defined quantity (called a bolus) at once.

Suitable for administration - a lyophilized formulation or solution that is appropriate to be given as a therapeutic agent.

Zymogen - protein C zymogen, as used herein, refers to secreted, inactive forms, whether one chain or two chains, of protein C.

Pharmaceutically acceptable buffer - a pharmaceutically acceptable buffer is known in the art. Pharmaceutically acceptable buffers include sodium phosphate, sodium citrate, sodium acetate, or TRIS.

Activated protein C is an antithrombotic agent with a wider therapeutic index than available anticoagulants, such as heparin and the oral hydroxycoumarin type anticoagulants. As an antithrombotic agent, aPC has a profound effect on the treatment of a wide variety of acquired disease states involving intravascular coagulation, including thrombotic stroke, deep vein thrombosis, pulmonary embolism, peripheral arterial thrombosis, emboli originating from the heart or peripheral arteries, acute myocardial infarction, disseminated intravascular coagulation, and acute pre or postcapillary occlusions, including transplantations or retina thrombosis.

The present invention relates to formulations of activated protein C. The desired formulation would be one that is a stable lyophilized product of high purity consisting of activated protein C and a bulking agent selected from the group consisting of mannitol, trehalose, raffinose, and sucrose. The lyophilized product is reconstituted with the appropriate diluent such as sterile water or sterile saline. Preferably, the resulting solution has a pH of about 5.5 to about 6.5.

The molecular interactions in a formulation between activated protein C, buffer, salt concentration, pH, temperature, and bulking agents, are complex, and the role that each factor contributes to the stability of the formulation is unpredictable. The lyophilized formulations of the present invention provide stable, enzymatically active, activated protein C upon resuspension because of reduced autodegradation. The present invention has particularly reduced levels of des(1-9) aPC and des(1-10) aPC. Generally, the levels of des(1-9) and des(1-10) aPC are less than 10% of the autodegradation product. Preferably, the levels of des(1-9) and des(1-10) aPC are

●   ● PCT/US98/08386

-6-

less than 8% of the autodegradation product. Still more preferably, the levels of des(1-9) and des(1-10) aPC are less than 5% and most preferably less than 3% of the autodegradation product. This stability is obtained through

5   careful control of the processing conditions and by the addition of sucrose, trehalose, raffinose, or mannitol. Interestingly, other bulking agents such as hydroxyethyl starch and glycine do not offer the necessary stability or pharmaceutical elegance.

10   The bulking agents of the present invention provide a pharmaceutically elegant formulation which has a uniform appearance and is readily solubilized when resuspended with the appropriate solute. Upon reconstitution, the formulation is stable for up to 24 hours to 48 hours at room

15 temperature. Resulting in stability previously unachievable.

         Preferred bulking agents in the formulation of activated protein C are sucrose, trehalose and raffinose. More preferred bulking agents are sucrose and raffinose and

20 the most preferred bulking agent is sucrose. The amount of bulking agent in the formulation is 1 part aPC to 1 to 10 parts bulking agent on a weight to weight basis. Moreover, the bulking agent concentration of the formulation is an important formulation variable of the freeze drying process.

25 The optimum concentration of bulking agent is dependent on the amount of aPC and species of bulking agent selected. The preferred concentration of sucrose in the freezing solution is 10 to 40 mg/mL. A more preferred concentration of sucrose is 15 to 30 mg/mL. The most preferred

30 concentration of sucrose in the freezing solution is 15 mg/mL in a formulation of aPC at 2.5 mg/mL. The most preferred concentration of sucrose in the freezing solution is 30 mg/mL in a formulation of aPC at 5.0 mg/mL. The presence of the claimed bulking agent in the formulation of

35 activated protein C offers increased chemical and physical stability.

SUBSTITUTE SHEET (RULE 26)

Prior to freeze drying and upon reconstitution, it is
preferable to maintain the pH in the range of 5.5 to 6.5 to
minimize solution state autodegradation. The preferred pH
of the formulation is a pH between about pH 5.6 and about pH
5 6.4. More preferred is a pH between about 5.7 to about 6.3.
Even more preferred is a pH between about 5.8 to about 6.2.
Still even more preferred is a pH between about 5.9 to about
6.1. The most preferred pH is about pH 6.0.

To maintain effective pH control, the aPC solution
10 should contain a pharmaceutically acceptable buffer.
Accordingly, upon freeze-drying, the formulation optionally
and preferably comprises a pharmaceutically acceptable
buffer. Representative buffer systems include Tris-acetate,
sodium citrate, and sodium phosphate. More preferred buffer
15 systems include sodium citrate and sodium phosphate. The
most preferred buffer is sodium citrate. The preferred
molarity of the buffer system is 10 mM to 50 mM. A more
preferred molarity of the buffer system is 10 mM to 20 mM.
The most preferred molarity is 40 mM. The skilled artisan
20 will recognize that many other buffer systems are available
which also can be used in the formulations of the present
invention.

Similarly, during freeze drying and upon
reconstitution, the ionic strength is a critical variable to
25 ensure solution state stability. The ionic strength is
generally determined by the salt concentration of the
solution. Pharmaceutically acceptable salts typically used
to generate ionic strength include but are not limited to
potassium chloride (KCl) and sodium chloride (NaCl). The
30 preferred salt in the present invention is sodium chloride.
During freeze-drying, the salt concentration must be high
enough to cause the salt to crystallize during the freezing
step of the freeze-drying cycle. Preferably, the sodium
chloride concentration is greater than 150 mM. More
35 preferably, the sodium chloride concentration in the
freezing solution is between 150 mM to 1000 mM. For a
formulation containing 2.5 mg/mL aPC, the more preferable

sodium chloride concentration in the freezing solution is between 150 mM to 650 mM. Even more preferably the sodium chloride concentration in the freezing solution is between 250 mM to 450 mM. Still even more preferably the sodium

5 chloride concentration in the freezing solution is between 300 mM to 400 mM. The most preferable sodium chloride concentration in the freezing solution is 325 mM for a formulation containing 2.5 mg/mL aPC.

Similarly, for a formulation containing 5.0 mg/mL aPC,

10 the more preferable sodium chloride concentration in the freezing solution is between 150 mM to 1000 mM. Even more preferably the sodium chloride concentration in the freezing solution is between 250 mM to 750 mM. Still even more preferably the sodium chloride concentration in the freezing

15 solution is between 400 mM to 700 mM. The most preferable sodium chloride concentration in the freezing solution is 650 mM for a formulation containing 5.0 mg/mL aPC.

The ratio of aPC:salt:bulking agent (w:w:w) is an important factor in a formulation suitable for the freeze

20 drying process. The ratio varies depending on the concentration of aPC, salt selection and concentration and bulking agent selection and concentration. One skilled in the art could readily identify the preferred ratio of aPC:salt:bulking agent by techniques appreciated in the art

25 and described, for example, in Example 1. Particularly, a weight ratio of one part activated protein C to between about 7 to 8 parts salt to between about 5 to 7 parts bulking agent is preferred. More preferred is a weight ratio of one part activated protein C to between about 7.5

30 to about 8 parts salt to between about 5.5 to about 6.5 parts bulking agent. Most preferred is a ratio of about 1 part activated protein C to about 7.6 parts salt to about 6 parts bulking agent.

The preferred salt is sodium chloride at a

35 concentration of 325 mM (for a formulation containing 2.5 mg/mL aPC) and 650 mM (for a formulation containing

5.0 mg/mL aPC) and at a ratio of about 1.3:1 with sucrose
(w:w). This concentration is high enough to cause the salt
to crystallize during the freezing process, most likely
resulting in an amorphous mixture of aPC, sucrose, and
5   citrate that can be lyophilized. Thus, the ionic strength
of NaCl at the preferred concentrations of 325 mM and 650 mM
convey a stability to the formulation during the
freeze-drying process.

The present invention further provides a process for
10 preparing a stable lyophilized formulation which comprises
lyophilizing a solution comprising activated protein C and a
bulking agent selected from the group consisting of
mannitol, trehalose, raffinose, and sucrose, and mixtures
thereof. The invention also provides a process for
15 preparing a stable lyophilized formulation which comprises
lyophilizing a solution comprising about 2.5 mg/mL activated
protein C, about 15 mg/mL sucrose, about 19 mg/mL NaCl, and
a sodium citrate buffer having a pH greater than 5.5 but
less than 6.5. Furthermore, the present invention provides
20 a process for preparing a stable lyophilized formulation
which comprises lyophilizing a solution comprising about 5
mg/mL activated protein C, about 30 mg/mL sucrose, about 38
mg/mL NaCl, and a citrate buffer having a pH greater than
5.5 but less than 6.5.

25   The present invention provides a unit dosage form
comprising a unit dosage receptacle containing a stable
lyophilized formulation comprising activated protein C and a
bulking agent selected from the group consisting of
mannitol, trehalose, raffinose, and sucrose, and mixtures
30 thereof. Furthermore, the present invention provides a
method of treating disease states involving intravascular
coagulation comprising the administration of said
formulation.

The aPC is preferably administered parenterally to
35 ensure its delivery into the bloodstream in an effective
form by injecting the appropriate dose as continuous
infusion for about one to about forty-eight hours. The

amount of aPC administered is from about 0.01 mg/kg/hr to
about 0.05 mg/kg/hr. Alternatively, the aPC will be
administered by injecting a portion of the appropriate dose
per hour as a bolus injection over a time from about 5
5   minutes to about 30 minutes, followed by continuous infusion
of the appropriate dose for about twenty-three hours to
about 47 hours which results in the appropriate dose
administered over 24 hours to 48 hours.

       The following examples will help describe how the
10   invention is practiced and will illustrate the invention.
The scope of the present invention is not to be construed as
merely consisting of the following examples.

Preparation 1

15

Preparation of Human Protein C

       Recombinant human protein C (r-hPC) was produced in
Human Kidney 293 cells by techniques well known to the
skilled artisan such as those set forth in Yan, U.S. Patent
No. 4,981,952, the entire teaching of which is herein
20   incorporated by reference. The gene encoding human protein
C is disclosed and claimed in Bang, *et al.*, U.S. Patent No.
4,775,624, the entire teaching of which is incorporated
herein by reference. The plasmid used to express human
protein C in 293 cells was plasmid pLPC which is disclosed
25   in Bang, *et al.*, U.S. Patent No. 4,992,373, the entire
teaching of which is incorporated herein by reference. The
construction of plasmid pLPC is also described in European
Patent Publication No. 0 445 939, and in Grinnell, *et al.*,
1987, Bio/Technology 5:1189-1192, the teachings of which are
30   also incorporated herein by reference. Briefly, the plasmid
was transfected into 293 cells, then stable transformants
were identified, subcultured and grown in serum-free media.
After fermentation, cell-free medium was obtained by
microfiltration.

35   The human protein C was separated from the culture
fluid by an adaptation of the techniques of Yan, U.S. Patent
No. 4,981,952. The clarified medium was made 4 mM in EDTA

before it was absorbed to an anion exchange resin (Fast-Flow
Q, Pharmacia). After washing with 4 column volumes of 20 mM
Tris, 200 mM NaCl, pH 7.4 and 2 column volumes of 20 mM
Tris, 150 mM NaCl, pH 7.4, the bound recombinant human
5   protein C zymogen was eluted with 20 mM Tris, 150 mM NaCl,
10 mM $CaCl_2$, pH 7.4. The eluted protein was greater than
95% pure after elution as judged by SDS-polyacrylamide gel
electrophoresis.

    Further purification of the protein was accomplished by
10 making the protein 3 M in NaCl followed by adsorption to a
hydrophobic interaction resin (Toyopearl Phenyl 650 M,
TosoHaas) equilibrated in 20 mM Tris, 3 M NaCl, 10 mM $CaCl_2$,
pH 7.4. After washing with 2 column volumes of
equilibration buffer without $CaCl_2$, the recombinant human
15 protein C was eluted with 20 mM Tris, pH 7.4.

    The eluted protein was prepared for activation by
removal of residual calcium. The recombinant human protein
C was passed over a metal affinity column (Chelex-100, Bio-
Rad) to remove calcium and again bound to an anion exchanger
20 (Fast Flow Q, Pharmacia). Both of these columns were
arranged in series and equilibrated in 20 mM Tris, 150 mM
NaCl, 5 mM EDTA, pH 7.4. Following loading of the protein,
the Chelex-100 column was washed with one column volume of
the same buffer before disconnecting it from the series.
25 The anion exchange column was washed with 3 column volumes
of equilibration buffer before eluting the protein with 0.4
M NaCl, 20 mM Tris-acetate, pH 6.5. Protein concentrations
of recombinant human protein C and recombinant activated
protein C solutions were measured by UV 280 nm extinction
30 $E^{0.1\%}=1.81$ or 1.85, respectively.

<u>Preparation 2</u>
<u>Activation of Recombinant Human Protein C</u>
    Bovine thrombin was coupled to Activated CH-Sepharose
35 4B (Pharmacia) in the presence of 50 mM HEPES, pH 7.5 at
4°C. The coupling reaction was done on resin already packed

into a column using approximately 5000 units thrombin/mL
resin. The thrombin solution was circulated through the
column for approximately 3 hours before adding 2-amino-
ethanol (MEA) to a concentration of 0.6 mL/L of circulating
5   solution. The MEA-containing solution was circulated for an
additional 10-12 hours to assure complete blockage of the
unreacted amines on the resin. Following blocking, the
thrombin-coupled resin was washed with 10 column volumes of
1 M NaCl, 20 mM Tris, pH 6.5 to remove all non-specifically
10   bound protein, and was used in activation reactions after
equilibrating in activation buffer.

Purified r-hPC was made 5 mM in EDTA (to chelate any
residual calcium) and diluted to a concentration of 2 mg/mL
with 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5. This
15   material was passed through a thrombin column equilibrated
at 37°C with 50 mM NaCl and either 20 mM Tris pH 7.4 or
20 mM Tris-acetate pH 6.5. The flow rate was adjusted to
allow for approximately 20 min. of contact time between the
r-hPC and thrombin resin. The effluent was collected and
20   immediately assayed for amidolytic activity. If the
material did not have a specific activity (amidolytic)
comparable to an established standard of aPC, it was
recycled over the thrombin column to activate the r-hPC to
completion. This was followed by 1:1 dilution of the
25   material with 20 mM buffer as above, with a pH of either 7.4
or 6.5 to keep the aPC at lower concentrations while it
awaited the next processing step.

Removal of leached thrombin from the aPC material was
accomplished by binding the aPC to an anion exchange resin
30   (Fast Flow Q, Pharmacia) equilibrated in activation buffer
(either 20 mM Tris, pH 7.4 or 20 mM Tris-acetate, pH 6.5)
with 150 mM NaCl. Thrombin does not interact with the anion
exchange resin under these conditions, but passes through
the column into the sample application effluent. Once the
35   aPC is loaded onto the column, a 2-6 column volume wash with
20 mM equilibration buffer is done before eluting the bound
aPC with a step elution using 0.4 M NaCl in either 5 mM

Tris-acetate, pH 6.5 or 20 mM Tris, pH 7.4. Higher volume washes of the column facilitated more complete removal of the dodecapeptide. The material eluted from this column was stored either in a frozen solution (-20°C) or as a

5   lyophilized powder.

The anticoagulant activity of activated protein C was determined by measuring the prolongation of the clotting time in the activated partial thromboplastin time (APTT) clotting assay. A standard curve was prepared in dilution

10   buffer (1 mg/mL radioimmunoassay grade bovine serum albumin [BSA], 20 mM Tris, pH 7.4, 150 mM NaCl, 0.02% NaN_3) ranging in protein C concentration from 125-1000 ng/mL, while samples were prepared at several dilutions in this concentration range. To each sample cuvette, 50 μL of cold horse plasma and 50 μL of reconstituted activated partial

15   thromboplastin time reagent (APTT Reagent, Sigma) were added and incubated at 37°C for 5 min. After incubation, 50 μL of the appropriate samples or standards were added to each cuvette. Dilution buffer was used in place of sample or standard to determine basal clotting time. The timer of the

20   fibrometer (CoA Screener Hemostasis Analyzer, American Labor) was started immediately after the addition of 50 μL 37°C 30 mM $CaCl_2$ to each sample or standard. Activated protein C concentration in samples are calculated from the linear regression equation of the standard curve. Clotting

25   times reported here are the average of a minimum of three replicates, including standard curve samples.

<u>Example 1</u>
<u>Formulation of Activated Protein C</u>

30   The human activated protein C was prepared as described in Preparations 1 and 2. The activated protein C formulations were analyzed for processing in a conventional freeze dryer. Freeze-Drying Microscopy and differential

35   Scanning Calorimetry (DSC) were used to measure two parameters that determine if a formulation can be processed

in a conventional freeze dryer. Freeze-Dry Microscopy is a
useful technique in determining the collapse temperatures of
the frozen solutions that are to be lyophilized. DSC is a
useful technique in determining the glass-transition
5   temperature (Tg') of the frozen solution. The collapse and
glass-transition temperatures are especially helpful in
predicting the upper temperature limits that can be safely
used during the freeze-drying process. Results of Freeze-
Drying Microscopy are complimentary to the glass-transition
10 temperature of the Tg', values obtained by DSC. A collapse
temperature above -40°C is optimal for the sample to be
processed in a conventional freeze-dryer.

Table 1:   Freeze dry processing of aPC formulation matrices

15

Formulation Matrix			
aPC Conc.	Sucrose Conc.	NaCl Conc.	Collapse Temperature
2.5 mg/mL	15 mg/mL	50 mM	-59°C
2.5 mg/mL	15 mg/mL	150 mM	-60°C
2.5 mg/mL	15 mg/mL	325 mM	-37°C
5.0 mg/mL	30 mg/mL	50 mM	-50°C to -45°C
5.0 mg/mL	30 mg/mL	150 mM	-60°C to -55°C
5.0 mg/mL	30 mg/mL	325 mM	-64°C
5.0 mg/mL	30 mg/mL	650 mM	-32°C to -28°C

The ratio of aPC to sucrose to sodium chloride (in 10
or 20 mM citrate buffer) is an important formulation
variable affecting the collapse and glass-transition
20 temperatures. To be processed in a conventional freeze-
dryer, the sodium chloride concentration must be high enough
(preferably 325 mM for 2.5 mg/mL aPC and 650 mM for 5 mg/mL
aPC formulations) to cause the sodium chloride to
crystallize-out during the freezing part of the freeze-
25 drying process. Formulations of aPC can be processed in a
conventional freeze dryer to produce lyophilized products

consisting of 1 part aPC, 6 parts sucrose, and 7.6 parts
sodium chloride by weight.

Example 2
Stability of aPC in Product Formulations
Containing Different Bulking Agents

Formulations of aPC were prepared to investigate the
effect of various bulking agents on the stability of the
molecule. A total of six excipients were added to aPC in
phosphate buffer containing no salt. These bulking agents
are glycine, mannitol, sucrose, trehalose, raffinose, and
hydroxyethyl starch (HES). The stability of aPC in the
phosphate, no salt, no bulking agent formulation ("control")
was compared to that in the bulking agent formulations.
Samples were stored at 50°C, 40°C, and 25°C for various
lengths of time. Data from analyses of these samples were
compared to the initial values (time =0). APTT potency,
size exclusion-high performance liquid chromatography
(SE-HPLC), SDS-PAGE, and protein content assays were used to
evaluate the physical and chemical stability of the
formulations.

Formulations of aPC were prepared by dissolving aPC in
phosphate buffer to 5 mg/mL aPC. Bulking agents were added
to portions of the aPC solution at a ratio of 6:1 (bulking
agents to aPC), or 30 mg/mL. The samples were lyophilized
to 5 mg aPC/vial.

The formulations were put on stability at 50°C for 14
and 28 days; 40°C for 28 days, 48 days and 6 months; and
25°C for 6 and 12 months. For each time point, two vials of
each formulation were analyzed independently as separate
samples and data from these samples were compared to those
from initial values (time =0). Analyses included aPC
potency (APTT), SDS-PAGE, percent of aPC monomer, and
protein content.

control

		25°C		50°C			40°C			
	Vial	Initial	6 month	12 month	Initial	14 day	28 day	Initial	28 day	84 day	6 month
APTT Potency (U/mg)	1	321	294	236	321	248	248	321	248	221	215
	2	321	251	242	321	245	227	321	279	233	176
Monomer Content (%)	1	99.3	98.3	96.5	99.3	97.5	97.0	99.3	97.7	96.2	95.1
	2	99.2	95.8	96.4	99.2	97.3	97.1	99.2	97.7	96.1	95.4

glycine

		25°C		50°C			40°C			
	Vial	Initial	6 month	12 month	Initial	14 day	28 day	Initial	28 day	84 day	6 month
APTT Potency (U/mg)	1	282	233	142	282	164	97	282	191	155	158
	2	321	239	191	321	161	142	321	215	152	79
Monomer Content (%)	1	99.1	98.4	93.3	99.1	97.4	97.2	99.1	97.8	96.4	95.8
	2	99.1	98.4	96.3	99.1	97.3	97.1	99.1	97.7	96.4	95.7

mannitol

	Vial	25°C			50°C			40°C			
		Initial	6 month	12 month	Initial	14 day	28 day	Initial	28 day	84 day	6 month
APTT Potency (U/mg)	1	309	227	255	309	270	245	309	273	270	282
	2	321	321	267	321	239	242	321	300	251	191
Monomer Content (%)	1	99.2	98.8	97.4	99.2	98.2	98.1	99.2	98.4	97.6	97.8
	2	99.2	98.7	97.6	99.2	98.2	98.0	99.2	98.4	97.6	97.8

sucrose

	Vial	25°C			50°C			40°C			
		Initial	6 month	12 month	Initial	14 day	28 day	Initial	28 day	84 day	6 month
APTT Potency (U/mg)	1	327	300	288	327	300	288	327	267	306	285
	2	297	300	306	297	291	291	297	321	242	294
Monomer Content (%)	1	99.2	99.0	98.5	99.2	98.7	98.9	99.2	98.8	98.5	98.9
	2	99.2	99.0	98.5	99.2	98.7	98.9	99.2	98.8	98.5	98.9

trehalose

	Vial	Initial	25°C 6 month	25°C 12 month	Initial	50°C 14 day	50°C 28 day	Initial	40°C 28 day	40°C 84 day	40°C 6 month
APTT Potency (U/mg)	1	312	291	282	312	258	282	312	273	276	276
	2	309	315	282	309	270	215	309	303	245	255
Monomer Content (%)	1	99.2	99.0	98.4	99.2	98.6	98.8	99.2	98.8	98.4	98.7
	2	99.2	98.8	98.4	99.2	98.6	98.8	99.2	98.7	98.4	98.7

raffinose

	Vial	Initial	25°C 6 month	25°C 12 month	Initial	50°C 14 day	50°C 28 day	Initial	40°C 28 day	40°C 84 day	40°C 6 month
APTT Potency (U/mg)	1	321	270	255	321	261	258	321	276	273	279
	2	288	285	306	288	255	264	288	270	239	255
Monomer Content (%)	1	99.1	99.0	97.0	99.1	98.6	98.7	99.1	98.7	98.4	98.6
	2	99.1	99.0	98.2	99.1	98.6	98.7	99.1	98.7	98.4	98.6

HES

	Vial	25°C Initial	25°C 6 month	25°C 12 month	50°C Initial	50°C 14 day	50°C 28 day	40°C Initial	40°C 28 day	40°C 84 day	40°C 6 month
APTT Potency (U/mg)	1	282	188	176	282	182	164	282	194	185	145
	2	285	245	215	285	188	161	285	176	152	103
Monomer Content (%)	1	97.8	95.6	92.2	97.8	93.0	91.8	97.8	93.7	90.6	88.7
	2	97.8	95.3	91.8	97.8	92.9	91.0	97.8	92.9	90.5	88.5

-20-

There were no significant changes in pH, color, package characteristics and physical appearance for any of the samples over the one year stability time period. When analyzed by the APTT and SE-HPLC procedures, the HES and glycine formulation had less physical stability (through aggregation) and chemical stability (potency) when compared to the control. The mannitol formulation offered slightly better physical and chemical stability than the control, and the remaining formulations, sucrose, trehalose and raffinose, all demonstrated even more superior physical and chemical stability when compared to the control. Therefore, mannitol sucrose, trehalose and raffinose, as bulking agents in aPC formulations, offer increased chemical and physical stability when compared to an aPC formulation without a bulking agent or those having glycine or HES.

Example 3
Stability of Recombinant Human Activated Protein C

Two lots of a lyophilized formulation of recombinant human activated protein C (aPC) were stored for 1 month at 40°C/75% relative humidity, and then analyzed for possible degradation. The stability of aPC was also monitored after reconstitution with sterile water and storage for up to 72 hours at ambient temperature. The lyophilized aPC product consisted of 10 mg aPC, 60 mg sucrose, 76 mg sodium chloride, and 15.1 mg citrate per vial. The aPC in this formulation is stable in the dry state for at least one month when stored at 40°C/75% relative humidity, and in solution for 24 hours when stored at ambient temperature.

Both lots were prepared using the same unit formula of 10 mg aPC, 60 mg sucrose, 76 mg sodium chloride, and 15.1 mg citrate per vial. Both lyophilized lots of aPC were stored for 1 month at 40°C/75% relative humidity and the stability of aPC was monitored using the APTT potency assay, ion-pairing HPLC for quantitation of aPC peptides and mass spectrometry for quantitation of protein variant forms. One

lot was also reconstituted with sterile water, to 1 mg/mL
aPC, and held at ambient temperature. The stability of aPC
in solution was monitored at the 0, 1, 4, 8, 24, 48 and
72 hour time points using the APTT and mass spectrometry
5   methods.

There was no loss of aPC activity and an insignificant
amount of structural degradation of the molecule after
storage in the dry state for one month at 40°C/75% relative
humidity. The aPC in this formulation is stable for up to
10   24 hours at 1 mg/mL after reconstitution.

-22-

We claim:

1. A stable lyophilized formulation comprising activated protein C and a bulking agent selected from the group consisting of mannitol, trehalose, raffinose, and sucrose, and mixtures thereof.

2. The formulation of Claim 1, wherein the bulking agent is sucrose, trehalose or raffinose.

3. The formulation of Claim 2, wherein the bulking agent is sucrose or raffinose.

4. The formulation of Claim 3, wherein the bulking agent is sucrose.

5. The formulation of Claim 1, which further comprises a pharmaceutically acceptable salt.

6. The formulation of Claim 5 wherein the weight ratio is about 1 part activated protein C, between about 7 to 8 parts salt, and between about 5 to 7 parts bulking agent.

7. The formulation of Claim 6 wherein the weight to weight ratio is about 1 part activated protein C, between about 7.2 to 7.8 parts salt and between about 5.5 to 6.5 parts bulking agent.

8.   The formulation of Claim 7 wherein the weight to weight ratio is about 1 part activated protein C, about 7.6 parts salt and about 6 parts bulking agent.

5   9.   The formulation of Claim 8 wherein the salt is NaCl.

10.   The formulation of Claim 9 wherein the bulking agent is sucrose.

10

11.   The formulation of Claim 1, which further comprises a pharmaceutically acceptable buffer.

12.   The formulation of Claim 11, wherein said buffer
15   is selected from Tris-acetate, sodium citrate, or sodium phosphate.

13.   The pharmaceutically acceptable buffer of Claim 12, wherein said buffer is sodium citrate.

20

14.   A stable lyophilized formulation comprising about 2.5 mg/mL activated protein C, about 15 mg/mL sucrose, and about 20 mg/mL NaCl.

25   15.   A stable lyophilized formulation comprising about 5 mg/mL activated protein C, about 30 mg/mL sucrose, and about 38 mg/mL NaCl.

-24-

16. A process for preparing the formulation of Claim 1, which comprises lyophilizing a solution comprising activated protein C and a bulking agent selected from the group consisting of mannitol, trehalose, raffinose, and sucrose and mixtures thereof.

17. The process of Claim 16, wherein the bulking agent is sucrose, trehalose or raffinose.

18. The process of Claim 17, wherein the bulking agent is sucrose or raffinose.

19. The process of Claim 18, wherein the bulking agent is sucrose.

20. The process of Claim 16, which further comprises a pharmaceutically acceptable salt.

21. A process of Claim 20 wherein the weight ratio is about 1 part activated protein C, between about 7 to 8 parts salt and between about 5 to 7 parts bulking agent.

22. The process of Claim 21 wherein the weight to weight ratio is about 1 part activated protein C, between about 7.2 to 7.8 parts salt and between about 5.5 to 6.5 parts bulking agent.

23. The process of Claim 22 wherein the weight to weight ratio is about 1 part activated protein C, about 7.6 parts salt and about 6 parts bulking agent.

5

24. The process of Claim 16, wherein the pH of the solution is between 5.5 to 6.5.

25. The process of Claim 24 wherein the pH is about 5.8 to 6.2.

10

26. The process of Claim 25 wherein the pH is about 6.0.

27. A process for preparing a stable lyophilized

15 formulation which comprises lyophilizing a solution comprising about 2.5 mg/mL activated protein C, about 15 mg/mL sucrose, about 20 mg/mL NaCl, and a citrate buffer, said solution having a pH greater than 5.8 but less than 6.2.

20

28. A process for preparing a stable lyophilized formulation which comprises lyophilizing a solution comprising about 5 mg/mL activated protein C, about 30 mg/mL sucrose, about 38 mg/mL NaCl, and a citrate buffer, said

25 solution having a pH greater than 5.8 but less than 6.2.

29. A unit dosage form comprising a unit dosage receptacle containing the formulation of Claim 1.

-26-

30. A unit dosage form comprising a unit dosage receptacle containing the formulation of Claim 8.

31. A method of treating acquired disease states involving intravascular coagulation comprising administering in patients in need thereof the formulation of Claim 1.

32. A method of treating acquired disease states involving intravascular coagulation comprising administering in patients in need thereof the formulation of Claim 14.

33. A method of treating acquired disease states involving intravascular coagulation comprising administrating in patients in need thereof the formulation of Claim 15.

34. A stable lyophilized formulation of any one of Claims 1 through 12 for use as a medicament in the treatment of disease states involving intravascular coagulation.

35. A stable lyophilized formulation of any one of Claims 1 through 12 for use as a medicament in the treatment of thrombotic stroke.

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/725
US CL :514/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/02

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,849,403 A (STOCKER et al) 18 JULY 1989, COL. 14, lines 5-32	1-35
Y	EP 03 260 14 A1 (HOECHST JAPAN LIMITED), 08 February 1989, pages 3, 4.	1-35
Y	WANG Y.J. et al. Parenteral Formulations of Proteins and Peptides. Stability and Stabilizers. J. Parenteral Science and Technology, 1988, Vol. 42, Number 2S, pages S3-S26, especially pages S16, S21	1-35

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	
"P"	document published prior to the international filing date but later than the priority date claimed	"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
01 JULY 1998	0 3 SEP 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	MICHAEL BORIN
Facsimile No. (703) 305-3230	Telephone No. (703) 305-4506

Form PCT/ISA/210 (second sheet)(July 1992)*

THIS PAGE BLANK (USPTO)

BO

OPI DATE 12/04/99
AOJP DATE 10/06/99

LN. ID
NUMBER PCT/AT98/00224

92449/98

AU9892449

(51) Internationale Patentklassifikation 6 :

A61K 38/48

A1

(11) Internationale Veröffentlichungsnummer: **WO 99/15196**

(43) Internationales
Veröffentlichungsdatum: 1. April 1999 (01.04.99)

(21) Internationales Aktenzeichen: PCT/AT98/00224

(22) Internationales Anmeldedatum: 17. September 1998
(17.09.98)

(30) Prioritätsdaten:
A 1591/97   19. September 1997 (19.09.97)   AT

(71) Anmelder (für alle Bestimmungsstaaten ausser US): IMMUNO ~~BAXTER~~
AKTIENGESELLSCHAFT [AT/AT]; Industriestrasse 67,
A-1221 Wien (AT).

(72) Erfinder; und
(75) Erfinder/Anmelder (nur für US): LINNAU, Yendra [AT/AT];
Lavendelweg 24, A-1224 Wien (AT). TURECEK,
Peter [AT/AT]; Hauptstrasse 59g, Weidling, A-3400
Klosterneuburg (AT). SCHWARZ, Hans-Peter [AT/AT];
Schindlergasse 32, A-1180 Wien (AT).

(74) Anwälte: SONN, Helmut usw.; Riemergasse 14, A-1010 Wien
(AT).

Gemeindeaugasse 3
A-1220 Wien
Austria

(81) Bestimmungsstaaten: AL, AM, AT, AU, AZ, BA, BB, BG,
BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB,
GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR,
KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN,
MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK,
SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW,
ARIPO Patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW),
eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ,
TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES,
FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI Patent
(BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE,
SN, TD, TG).

Veröffentlicht
Mit internationalem Recherchenbericht.
*Vor Ablauf der für Änderungen der Ansprüche zugelassenen
Frist; Veröffentlichung wird wiederholt falls Änderungen
eintreffen.*

(54) Title: PHARMACEUTICAL SUBSTANCE CONTAINING VARIOUS VITAMIN K-DEPENDENT FACTORS

(54) Bezeichnung: PHARMAZEUTISCHES PRÄPARAT ENTHALTEND VITAMIN K-ABHÄNGIGE EINZELFAKTOREN

(57) Abstract

    The invention relates to a pharmaceutical substance separated from a prothrombin complex, containing at least two different blood factors which are highly purified and vitamin k-dependent.

(57) Zusammenfassung

    Beschrieben wird ein pharmazeutisches separiertes Prothrombinkomplex-Präparat, das mindestens 2 hochgereinigte Vitamin K-abhängige Einzelblutfaktoren enthält.

A Pharmaceutical Preparation Comprising

Vitamin K-Dependent Single Factors

Abstract:

A pharmaceutical separated prothrombin complex
preparation is described which comprises at least two
highly purified, vitamin K-dependent single blood
factors.

A Pharmaceutical Preparation Comprising
Vitamin K-Dependent Single Factors

The invention relates to a pharmaceutical preparation comprising vitamin K-dependent single factors.

Vitamin K-dependent proteins are characterized in that they essentially require vitamin K for their biosynthesis. Thus, e.g., prothrombin (factor II) formed under the influence of vitamin K antagonists, in contrast to normal prothrombin can not bind Ca^{2+}. Normal prothrombin contains γ-carboxyglutamate at the N-terminal end, i.e. a second carboxyl group at the glutamate residue. For, in the course of biogenesis of functional prothrombin, the first ten glutamate residues in the amino-terminal region of the protein are carboxylated to γ-carboxyglutamate by a vitamin K-dependent enzyme system. This γ-carboxyglutamate group is a very strong chelating agent for calcium ions. Via these bound Ca^{2+} ions, prothrombin is bound on phospholipid membranes which are derived from cell membranes, e.g. from blood platelets, to thus obtain the correct topology for initiation of blood coagulation.

It is not only prothrombin which has γ-carboxyglutamate residues, but also the coagulation factors VII, IX and X are carboxylated on specific glutamate

- 1 -

residues to thus form a high affinity relative to calcium ions. Yet also further proteins involved in the coagulation cascade, such as protein C, protein S and protein Z, require vitamin K for their biosynthesis.

Vitamin K-dependent single factors, in particular factors II, VII, IX and X, have similar physical-chemical properties, such as, e.g., similar mol weights, pIs, electrophoretic mobility, etc. and therefore as a rule are recovered together as prothrombin complex (other designation: factor IX complex or PPSB-complex). On account of the similar protein characteristic, it is difficult to prepare the factors individually. The production of prothrombin complex preparations with a simultaneous isolation of all the factors contained therefore has always been preferred in the prior art over the production of blood factor concentrates when producing pharmaceutical preparations (cf. Brummelhuis in: Methods of Plasma Protein Fractionation, ed. Curling, 1980, Academic Press, pp. 117-128). Yet it has also been shown that the prothrombin complex factors, on account of their different stabilities or half-lives, respectively, can never be obtained in a physiological ratio (always 1 U of the protein) (cf. Müller et al., Krankenhausphar-mazie 13 (11), (1992), 528-531; Köhler et al., Thrombosis Research 60 (1990), pp. 63-70).

In EP-A 0 700 684, a prothrombin complex

concentrate together with at least one further blood-coagulation promoting component as an antidote for blood anticoagulants has been described.

There is the risk that prothrombin complex concentrates contain activated coagulation factors because they have been purified from complex protein mixtures, which activated coagulation factors in most instances are serine proteases. Yet particularly patients should not be coagulated, since the formation of thromboses may be or is, respectively, fatal. Even if only traces of these activated coagulation factors, in particular thrombin, are present in such a preparation, proteolytic inactivation of single factors will occur which, depending on the individual stability of the single factors, may have severe consequences. On the whole, thus, prothrombin complex concentrates tend to instabilities and are not suitable for an extended storage. These degradation reactions, in particular due to thrombin, occur even in the solid state, which means that also drying or lyophilisation, respectively, of the preparations will not result in a reliable storage stability.

Moreover, prothrombin complex concentrates are extremely unflexible as regards the relative ratios of the invididual factors contained. There are hardly any possibilities to influence these relative ratios in the course of purifying the prothrombin complex, which is

- 3 -

particularly disadvantageous if a prothrombin complex in which certain factors are enriched is desired.

Thus, it is an object of the present invention to provide pharmaceutical combination preparations of vitamin K-dependent proteins which are, or can be, respectively, precisely defined as regards their composition, which have a high stability, in particular during extended storage, and which are highly flexible as regards variation of their composition.

According to the invention, this object is achieved by a pharmaceutical separated prothrombin complex preparation comprising at least two chromatographically purified vitamin K-dependent single factors as active substances. Above all, the preparation according to the invention shall contain highly purified factor IX in combination with at least one further highly purified vitamin K-dependent single factor.

In contrast to the prior art in which the prothrombin complex has always been purified as a complex and has not been prepared by combination of single factors and, moreover, even at its best is present only up to an intermediary, i.e. moderate, purity, with the present invention a preparation is provided which contains the individual blood factors in highly purified form, which are freed from interfering contaminations, in particular of a thrombin activity. In particular, the single factors to be combined

- 4 -

according to the invention are purified by chromato-
graphic purification methods, such as ion exchange
chromatography, hydrophobic chromatography, affinity
chromatography and/or molecular exclusion chromato-
graphy, from plasma or recombinant cells. In this
manner, specific activities of at least 50% of the
theoretical purity, preferably at least 70%, in
particular at least 90%, up to the theoretical purity
can be attained for most vitamin K-dependent single
factors in each case. Accordingly, it is also preferred
to use factors which are substantially free (≤ 5%) of
denaturing products.

In the pharmaceutical production of blood
coagulation protein preparations, as a rule it is
differentiated between three different degrees of
purity: low, intermediary, and high.

Table 1

	In vivo Half-Life	Theoretical Purity
Factor II	60 h	0.1 U/mg
Factor VII	2 - 2.5 h	2000 (1667 - 2500)
Factor IX	18 - 24 h	250 (200 - 333)
Factor X	40 h	118 (100 - 143)

In particular when determining the factor II
activity, the results repeatedly are falsified because

- 5 -

of the presence of traces of factor IIa, since even traces of factor IIa will interfere with the concentration determination of factor II such that values even by a multiple higher than the theoretical purity can be determined. With factor VII, the values therefore are somewhat lower relative to the other factors, since factor VII is a very labile protein which is extremely rapidly converted to factor VIIa. Therefore, even a factor VII preparation which has more than 10% of the theoretical purity is considered as highly purified.

Since the preparation according to the invention is composed of single factor preparations which are strictly defined particularly as regards their activities and their degrees of purity, respectively, also the ratio of the single factors to each other can be optimally adjusted. Thus, also problems occurring in the prior art in the course of further processing of the total protein complex concentrates, e.g. by activity losses during virus inactivation, but also by processes which occur in the course of the purification procedure, can be avoided from the very beginning, since after combining the highly purified single factors to the preparation according to the invention, preferably no further processing steps will be carried out.

This means that the preparation according to the

invention on the whole has the advantage of being standardizable. In this manner it is ensured that the respective individual factors in the added concentration are contained in the preparation +/- 10% deviation.

According to the invention, preferred single factors are selected from the group consisting of factor II, factor VII, factor IX, factor X, protein C, protein S and protein Z. Preferred production methods for highly purified preparations of these proteins can be found e.g. in EP 0 796 623 (factors II and X), A 594/97 (factor VII), EP 0 496 725 (factor IX), EP 0 533 209 (protein C) and EP 0 406 216 (protein S).

In the preparation according to the invention, preferably at least the factor II, VII, IX and X, starting from highly purified single factors, are combined, wherein, optionally, also the highly purified single factors protein C, protein S and/or protein Z are admixed, so as to be able to provide a prothrombin complex which is as physiological as possible, i.e. a prothrombin complex whose composition corresponds to the physiological one - yet without the interfering accompanying proteins which are included during the purification of the prothrombin complex from plasma, such as, e.g., thrombin.

The single factors may be purified from plasma, in particular human plasma, or be prepared by recombinant

- 7 -

technology. Since in the preparation by recombinant DNA technology a separation of the structurally and physically-chemically very similar factors is not necessary, the preparation according to the invention preferably is combined of highly purified recombinant single factors. The factor may also be transgenically prepared; it may be a derivative, in particular a peptide, and/or a fragment.

The vitamin K-dependent single factors, factor II, factor VII, factor IX, factor X, protein S and C, have been cloned and sequenced, and their production has, e.g., been described in Falkner et al., Thrombosis and Haemostasis 68 (2) (1992), pp. 119 to 124, for vitamin K-dependent proteins.

According to the invention, the relative ratios of the highly purified single factors are easily adjustable in any desired relation to each other within the preparation, e.g. in that these ratios correspond to the natural ratios in blood, i.e. in that approximately per unit of the one factor, one unit each of the other factor is present. A preferred preparation according to the present invention therefore contains the highly purified single factors, factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (0.5 to 2) : (0.5 to 2) : (0.5 to 2). If also protein C, protein S and/or protein Z are present in the preparation, also

- 8 -

these single factors preferably have relative ratios of from 0.5 to 2.

On the other hand, it is also possible according to the invention to adjust the individual factors, due to their relative stabilities, in particular at the ratios of their relative half-lives, i.e. that more is provided of a less stable factor, and, correspondingly, less of the stable factor. In this instance, also the intended period of time of application or action, respectively, may additionally be taken into consideration, i.e. the longer this time period, the higher also the relative ratios have to be considered.

Also recombinant proteins of, e.g., changed half-lives, may then be standardized accordingly. Also further factors, such as, e.g., the in vivo recovery, may be included in a standardization of the factors.

A preparation which is preferred in this respect therefore comprises the single factors, factor II, factor VII, factor IX and factor X at relative ratios, based on international units, of (0.5 to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5), since the half-lives of prothrombin are 60 hours, of factor VII 2 hours, of factor IX 20 hours and of factor X 40 hours. A preferred preparation with these factors therefore comprises the single factors, factor II, factor VII, factor IX, factor X, protein C and protein S at relative ratios, based on international units, of (0.5

- 9 -

to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5) : (1 to 15) : (1 to 15).

Since factor II by far is the most stable one of these factors and, moreover, also in its activated form thrombin carries the highest stability risk, a preparation which does not contain prothrombin is preferred. Factor VII mostly is considered as rather unstable, and therefore in the preparation according to the invention it is preferably provided to an increased extent, e.g. in 10-fold concentration (based on international units). A particularly preferred preparation thus contains single factor VII and single factor II at a ratio of greater 10 : 1.

With the present preparation, preferably a prothrombin complex or a partial prothrombin complex is provided from highly purified single factors. In any event, it is preferred that the single factors in the preparation do not form a complex. This may be shown, e.g., by analytical ion exchange chromatography on Q-Sepharose (Pharmacia), wherein the single factors during elution with a salt gradient can be discretely eluted. In contrast thereto are the complexes as they occur in prothrombinase or in pro-prothrombinase.

Prothrombinase is an enzyme-substrate complex which forms on a phospholipid surface and enables the activation of prothrombin. Prothrombinase by definition consists of factor II (prothrombin), activated factor X

- 10 -

(factor Xa) cofactors V and/or Va, respectively, phospholipids and calcium ions. *In vivo*, these factors are present as a transient complex for the activation of prothrombin and the formation of thrombin. A corresponding pro-prothrombinase is defined as a complex of factors which are present at least partially modified or activated, respectively, for the formation of a prothrombinase. Pro-prothrombinase therefore is to be understood as a precursor of prothrombinase and as a complex in which one or more components are present in their precursors, as zymogens, or as proforms and which is formed on the basis of affinities of the components to each other.

For stability reasons, it is advantageous to avoid any presence of activated coagulation factors in the preparation according to the invention. A preferred embodiment therefore is characterized in that the preparation does not comprise any activated coagulation factors, in particular does not comprise any factor IIa, IXa, Xa and, optionally, VIIa.

Preferred preparations according to the invention comprise less than 0.1 U of factor VIII:C or factor VIII:Ag/mg of protein and/or less than 0.1 U of factor IIa/unit of prothrombin and/or less than 0.1 U of factor Xa/unit of factor X.

To maintain the excellent stability of the preparation according to the invention as long as

- 11 -

possible, it is advantageous to provide the preparation according to the invention in lyophilized form. In this manner it is possible to store the preparation according to the invention for an almost unlimited period of time and nevertheless reconstitute it as a lyophilisate within a short period of time to a ready-to-use solution.

According to a further preferred embodiment, the preparation according to the invention further comprises magnesium ions. These ions act competitively to calcium ions and can displace the calcium ions primarily in the complete or partial prothrombin complex. In this manner, a premature thrombin formation in a solution of the preparation according to the invention is prevented to an even higher extent and the latter thus is stabilized so much that even in an aqueous solution it will remain stable for many hours.

It has been shown that the pharmaceutical preparation according to the invention can even be provided as a stable infusion solution, primarily if it is ensured that it does not contain any free calcium ions. The content of free calcium ions can easily be determined by the known ion titration or by other analytical methods. To complex the calcium ions, e.g. a pharmaceutically acceptable chelating agent, preferably EDTA, and related substances, such as citrate, are suitable.

Preferably, the preparation according to the invention furthermore comprises antithrombin III in those amounts in which hitherto it has been used in stabilizing manner in prothrombin complex concentrates, optionally together with heparin. Although this measure does not seem absolutely necessary because of the high degree of purity of the single factors, it may be considered advantageous for pharmaceutical reasons or also for requirements of pharmacopoieas or other rules with a view to the prothrombin complex concentrates of the prior art.

In another preferred embodiment, the preparation therefore is free of albumin and/or stabilizers, such as in particular antithrombin III and/or heparin. In particular, the preparation according to the invention is also free from phospholipids.

According to a preferred embodiment, the pharmaceutical preparation according to the invention is freed from infectious viruses or other infectious agents as a consequence of a treatment for virus inactivation. This treatment for virus inactivation preferably is ensured by two independent virus inactivation or virus depletion methods.

Preferably, this inactivation treatment is ensured by a tenside and/or heat treatment, e.g. by a heat treatment in the solid state, in particular a vapor treatment according to EP-0 159 311, EP-0 519 901 or

- 13 -

EP-0 674 531.

Further treatments for virus inactivation also comprise the treatment with chemical or chemical/physical methods, e.g. with chaotropic substances according to WO 94/13329, DE 44 34 538 or EP-0 131 740 (solvent) or photoinactivation.

Nanofiltration or the antibody-intensified nanofiltration (WO 9740861) also constitute a preferred method for virus depletion within the scope of the present invention.

Preferably, the preparation according to the invention further comprises pharmaceutically acceptable buffer substances or stabilizers, e.g. the substances already provided for prothrombin complex concentrates in the pharmacopoeias.

A particularly preferred variant for ensuring the freedom from viruses of the product according to the invention consists in that it is composed of highly purified vitamin K-dependent single factors which in turn have already been virus-inactivated and optionally already have been freed from denaturing products being formed and from stabilizers, so that they can be present in virus-inactivated and nevertheless precisely defined form as regards their activities.

Since above all in thermal virus inactivation methods, partial inactivation processes of the prothrombin factors may occur which, depending on the

- 14 -

stability of the single factor, may lead to different yields and specific activities after the thermal treatment of prothrombin complex preparations, one embodiment of the method according to the invention also allows for adjusting the ratios in the mixture of single factors such that even after a thermal treatment, the factors will be present at the desired ratios to each other. For instance, if it is known that the thermal treatment of prothrombin does not result in a loss of activity, whereas the factors, factor X and factor IX also present in the combination preparation will be inactivated by 20% each, a mixed, thermally virus-inactivated "complex" can be adjusted by combining the factors II, IX and X at activity ratios of 1 : 1.25 : 1.25. After this adjustment of the ratios, virus inactivation may then be carried out, and from this a preparation will directly result which comprises these factors at the ratio of 1 : 1 : 1.

Preferably, factor X is used in its α-form and/or β-form.

A subject matter of the present invention is also a diagnostic preparation which according to the invention, is composed of the highly purified vitamin K-dependent single factors. Also for diagnosis, the advantages of definition, variability of concentration ratios and stability are of particular advantage.

The pharmaceutical preparation according to the

- 15 -

invention may, of course, be used for all other previous indications of the prothrombin complex.

Thus, a subject matter of the present invention is also the use of the preparation according to the invention for producing a preparation for the treatment of acquired or inherited blood coagulation disorders, for the treatment of severe hemorrhages, for the prophylaxis of hemorrhages, in particular if inherited blood coagulation disorders are present, for substitution therapy and for the treatment of hemophilia B.

Also for liver dysfunctions, the preparation according to the invention proves to be indicated.

When administered to a patient, the administration regimen has to be taken as a basis for dosage, wherein, however, the more precise definition of the preparations of the invention is advantageous.

The present invention will be explained in more detail by way of the following examples to which, however, it shall not be restricted.

E x a m p l e s :

E x a m p l e 1 : Production of the single factor preparations

1.1 Production of single factor preparations of plasmatic factor X and plasmatic factor II:

A lyophilized prothrombin complex factor preparation which contained factors II, IX, X as well

as protein C and protein S was prepared according to the method of Brummelhuis, H.G.J., Preparation of the Prothrombin complex. In: Methods of Plasma Protein Fractionation, Curling, J.M. ed., 117-128, Academic Press, New York, (1980), and heat-treated for virus inactivation according to EP 159 311. Accordingly, the lyophilisate (1,000 U of factor X/g, 1,200 U of factor II/g) was dissolved in distilled water so that the latter contained 50,000 U of factor X/l, and adjusted to pH 7.0. After additon of 12% (v/v) Tween® 80, it was stirred for 1 hour at room temperature. Subsequently, it was diluted 1:5 with a 20 mM Tris-HCl buffer, pH 7.0, and the prothrombin complex protein fraction was adsorbed on calcium phosphate ($Ca_3(PO_4)_2$) at a concentration of 30 g of $Ca_3(PO_4)_2$ per 1 of prothrombin complex solution by stirring for 1 h at room temperature. Subsequently, the solid phase was separated by centrifugation, 20 min at 5,000 rpm, and the precipitate was washed twice with 20 mM Tris-HCl buffer, pH 7.0, containing 10% ammonium sulfate, by resuspension and renewed centrifugation. A third washing was carried out in an analogous manner with 20 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl. Elution of the prothrombin complex fraction was effected with 1 M sodium phosphate solution, pH 7.0, wherein 25 ml of this solution per g of calcium phosphate were stirred for 1 hour at room temperature,

- 17 -

and subsequently the remaining precipitate was separated by centrifugation as above. Subsequently, the supernatant was subjected to an ammonium sulfate precipitation with 366 g of ammonium sulfate per 1 for 15 hours at 4°C under stirring. The precipitate containing the prothrombin complex fraction was separated by centrifugation as above. The precipitate was taken up in a 25 mM trisodium citrate dihydrate buffer containing 100 mM NaCl, 1 mM benzamidine hydrochloride, pH 6.0, and rebuffered on a column filled with Sephadex® G-25 at 4°C with a linear flow of 1 cm/min against 25 mM trisodium citrate dihydrate buffer containing 100 mM NaCl and 1 mM benzamidine hydrochloride, pH 6.0, so as to separate the ammonium sulfate. In doing so, the UV absorption at 280 nm and the electric conductivity were measured in the eluate flow. The protein-containing fractions were combined and subsequently subjected to an ion exchange chromatography over DEAE-Sepharose FF® (Pharmacia). The fractions were applied on a column (inner diameter : gel bed height = 1 : 1.3) with a gel volume of 8.2 l, 0.55 g of protein/l of gel, at a linear flow of 0.36 cm/min. Chromatography was carried out at 22°C. Before the proteins were applied, the column had been equilibrated with a 25 mM trisodium citrate dihydrate buffer, containing 100 mM NaCl, 1 mM benzamidine hydrochloride, pH 6.0. Elution of the protein fractions

was carried out in several steps with a buffer 1 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 245 mM NaCl, pH 6.0), buffer 2 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 270 mM NaCl, pH 6.0) and a buffer 3 (25 mM trisodium citrate dihydrate, 1 mM benzamidine hydrochloride, 400 mM NaCl, pH 6.0). Elution with buffer 1 was carried out with 2.4 column volumes, and in doing so, inert protein was separated. Elution was carried out with 5.6 column volumes in buffer 2, and here the fractions were collected which were analyzed for their contents of factor II, factor X, protein C and factor IX. The factor X-containing fractions which were free from factors II, IX and protein C were combined. This highly purified factor X preparation had a specific activity of 60 U/mg of protein.

By elution with buffer 3 (1.9 column volumes), factor II was desorbed, wherein, again, the fractions were collected and assayed for their contents of factor X, factor IX and factor II. The factor II-containing fractions were pooled. Both factor II and also the factor X-containing pool optionally could be subjected to an additional treatment for inactivation of pathogenic impurities by the addition of 1 M KSCN and incubation at 22°C for several hours.

The thus recovered factor II pool was adjusted to 1.8 M NaCl by the addition of sodium chloride, and the

- 19 -

pH was corrected to pH 7.0. Subsequently, this solution
was adsorbed on a gel, Phenylsepharose High
Performance® (Pharmacia), by hydrophobic interaction,
whereby 3 g of protein/1 of gel being bound. In a
column with a ratio of inner diameter : gel bed height
= 1 : 1.9, the protein fraction was adsorbed at a
linear flow of 0.25 cm/min, and subsequently was freed
from inert protein by washing with a buffer (25 mM
Tris-HCl, 3 M NaCl, pH 7.4). By gradient elution with
11.5 column volumes of 3 M - 0.9 M NaCl with
simultaneous collection of fractions, factor II was
eluted from the column. Those fractions which contained
factor II activity were pooled and yet were free from
factors X and IX. Subsequently, the collected factor
II-fractions were concentrated 10 times by
ultra/diafiltration via an ultrafiltration membrane
having a cut-off of 30 kD, and rebuffered against a
buffer containing 4 g of trisodium citrate dihydrate/1,
8 g of NaCl/1, pH 7.0. A thus prepared factor II-
preparation had a specific activity of 6.9 U/mg of
protein. Determination of the factor II activity was
carried out with the 1 step method, based on the
thromboplastin time, by using a factor II deficiency
plasma against the International Factor II Standard,
employing the reagent combination from BAXTER, Vienna.
In the coagulation analyses, other coagulation factors
were detectable in traces or were not detectable any

longer (factor VII < 0.00002 U/U factor II, factor IX 0.0002 U/U factor II, factor X 0.004 U/U factor II, protein C 0.003 U/U factor II, and factor VIII < 0.0002 U/U factor II).

As an alternative production method for a highly purified factor II, also a method was used in which first factor IX was separated from a lyophilized prothrombin complex factor preparation by hydrophobic chromatography, subsequently factor II was isolated, and the latter was highly purified by chromatography on hydroxyl apatite.

The prothrombin complex factor preparation was dissolved as described above and incubated with a detergent for 1 h at room temperature. Subsequently, a factor II, IX an X-containing fraction was isolated by ion exchange chromatography on DEAE Sepharose FF® (Pharmacia). From this, subsequently the factor IX-containing fraction was removed by interaction with Butyl-Toyopearl® (Toso Haas). The adsorption supernatant subsequently was purified on Phenyl-Sepharose High Performance® (Pharmacia) by a further hydrophobic interaction chromatography, wherein approximately 4 g of protein/l of gel could be adsorbed. In a column having a ratio of inner diameter : gel bed height = 1 : 1.9, the protein fraction was adsorbed at a linear flow of 0.25 cm/min, subsequently the inert protein was removed by washing with 20 mM

Tris-HCl, 3 M NaCl, pH 7.4, and finally the factor II-containing fraction was isolated by step-wise elution, which fraction desorbed from the gel at 1.9 M NaCl at decreasing conductivity. The factor II-containing fraction then was directly adsorbed on Ceramik-Hydroxylapatit® (BioRad). This was carried out on a column with a ratio of inner diameter : gel bed height = 1 : 4.8. Elution was effected at a linear flow of 3 cm/min. By elution with a salt gradient, factor II could be desorbed from the column. The factor II-containing fractions were collected and concentrated by ultra/diafiltration via polysulfone membranes having a cut-off of 30 kD, until the factor II-concentration was 50-100 U/ml. A thus-prepared factor II preparation had a specific activity of at least 7 U/mg protein. Other coagulation factors, in particular factor IX and factor VIII, were merely detectable in traces or not detectable at all. By selecting a suitable diafiltration buffer, the factor II preparation was transferred into a pharmaceutically acceptable buffer (e.g. 4 g trisodium citrate dihydrate/l, 8 g NaCl/l, pH 7.0).

1.2 Recovery of plasmatic factor VII:

30 ml of fresh frozen human citrated plasma were thawed at 0 - +4°C, and the cryoprecipitate incurred was separated by centrifugation at +2°C. The "cryosupernatant" resulting therefrom was admixed with

2 IU of heparin/ml. Subsequently, the proteins of the prothrombin complex were adsorbed with DEAE-Sephadex® A-50 (Pharmacia) at a concentration of 0.5 mg/ml. The gel-protein complex was separated from the solution and washed respectively with buffer 1 (4 g/l Na_3 citrate.$2H_2O$, 7 g/l NaCl, 9 g/l Na_2HPO_4.$2H_2O$, 500 IU heparin/l, pH 7.5) and subsequently with buffer 2 (4 g/l Na_3 citrate.$2H_2O$/l, 7 g/l NaCl, 500 IU heparin/l, pH 7.5).

In this manner, the prothrombin complex fraction comprising the coagulation factors prothrombin, small amounts of factor VII, factor IX and factor X was separated. The main portion of coagulation factor VII remaining in the supernatant after adsorption on DEAE-Sephadex® A50 was then recovered by adsorption on aluminum hydroxide. Thereto, 10 ml of a 2% aluminum-hydrogel suspension were admixed per 1 l of supernatant after the prothrombin complex has been separated, and it was stirred at 4°C for 30 minutes. Subsequently, the aluminum hydroxide-protein complex was separated by means of centrifugation at 5000 rpm for 10 minutes at approximately 4°C in a Sorvall RC3B Rotor H6000A. The supernatant was discarded, and the precipitate was suspended with 3.5% of the volume of the prothrombin complex supernatant used for adsorption in a solution of 4 g Na_3 citrate.$2H_2O$/l and 7 g NaCl/l, pH 7.5, and stirred for 30 minutes. In this manner the inert

- 23 -

protein was desorbed from aluminum hydroxide. Factor VII remaining on the aluminum hydroxide was pelletized by renewed centrifugation as described above. The supernatant was discarded, and the precipitate was used for further processing. For desorption of the protein fraction, the aluminum hydroxide factor VII complex was stirred for 30 minutes with 1 vol.-% of the prothrombin complex supernatant of an 0.3 mol/l phosphate buffer used for adsorption, pH 8.6 (53.4 g $Na_2HPO_4 \cdot 2H_2O/l$ were adjusted to pH 8.6 with a solution of 41.1 g $NaH_2PO_4 H_2O/l$) containing 1% TWEEN®80. For pathogen inactivation, subsequently TWEEN®-80 detergent was admixed to a final concentration of 15%, followed by stirring for 1 h at 40°C.

After the solution had been cooled to 22°C, one aliquot of 20 ml was rebuffered by means of column chromatography via Sephadex® G-50 (Pharmacia) against a solution of 4 g Na_3 citrate.$2H_2O$, pH 7.4, and diluted to the 10-fold with the same solution. Then the entire solution was applied onto a column which was packed with heparin sepharose CL6B (Pharmacia), having a diameter of 16 mm and a height of 10 cm. The flow rate was 1 ml/min. Subsequently, it was eluted with the 10-fold column volume with a salt gradient of from 0-1 M NaCl in a solution of 4 g Na_3 citrate.$2H_2O/l$, pH 7.4. The protein fraction was quantitated by measuring UV absorption at 280 nm. Simultaneously, fractions were

collected, and factor VII was determined therein with a chromogenic factor VII assay (Immunochrom Faktor VII:10, BAXTER AG, Vienna). The fractions of the eluted protein which contained factor VII were collected and combined. The elution volume of the factor VII fraction was 25 ml. In the protein fraction, the protein content was determined according to the method of Bradford, M.M. (Anal. Biochem. 72: 248-254, 1976), and factor VII as described above. Likewise, factor VIIa was quantitated according to the method of US 5,472,850 (Ser. No. 683682). From this it could be calculated that the eluting factor VII fraction had a specific activity of 98 units/mg of protein, while the factor VIIa content was below one unit/ml. Accordingly, a highly purified factor VII preparation without any substantial activation of factor VII could be recovered. The yield at the chromatography was more than 50%.

1.3 Plasmatic factor IX:

150 ml of prothrombin complex were pre-purified by means of dextrane sulfate (Miletich et al., Analytical Biochemistry 105, 304 (1980)). 20 ml of eluate (912 I.U. of factor IX, 160 I.U. of factor X) were applied on a column with 20 ml of an agarose polymer with octyl groups (Octyl-Sepharose-CL-4B (Pharmacia, Sweden)) with a flow rate of 360 ml/h. The column previously had been equilibrated with 80 ml of buffer A. After washing of

- 25 -

the loaded gel with 120 ml of buffer A, the factor IX-
containing fraction was eluted with 80 ml of a 250 mmol
NaCl solution.

The yield of factor IX was approximately 54% of the
starting activity. The specific activity was 186 I.U.
of factor IX/mg of protein. Factor X was only present
in traces any more.

1.4 Recovery of plasmatic protein C

Highly pure protein C was recovered from a crude
protein C fraction which was prepared from commercially
obtainable prothrombin complex concentrate.
Purification was carried out by affinity chromatography
by means of monoclonal antibodies. Monoclonal anti-
protein C antibody was prepared as follows:

BALB/C mice were immunized by intraperitoneal
injection with 100 μg of human protein C at two-week
intervals. After six weeks, once again 50 μg of the
human protein C were injected, and 3 days afterwards
fusion was carried out. The myeloma cell line (P3-X-63-
AG8-653, 1.5 x 10^7 cells) was mixed with 1.7 x 10^8
spleen cells from a mouse, and fusion was effected
according to a modified Köhler & Milstein method by
using PEG 1500 (Köhler G., Milstein C., Nature 256
(1975), 495-497).

Positive clones, tested by means of an ELISA, were
subcloned twice. Ascites production was effected by
injection of 5 x 10^6 hybridoma cells per BALB/C mouse

two weeks after Pristan treatment.

The immunoglobulin was purified from ascites by ammonium sulfate precipitation and subsequent chromatography by means of QAE-Sephadex and, thereafter, chromatography on Sephadex G200. To reduce the risk of transmitting murine viruses, the antibody was additionally subjected to a virus inactivation step prior to immobilisation. The thus obtained monoclonal antibodies against protein C were coupled to CNBR-Sepharose 4B (Pharmacia). To purify the protein C by means of affinity chromatography, the following buffers were used:

as adsorption buffer: 20 mM Tris, 2 mM EDTA, 0.25 M NaCl and 5 mM benzamidine;

as washing buffer, 20 mM Tris, 1 M NaCl, 2 mM benzamidine, 2 mM EDTA was used; the pH was 7.4;

as elution buffer, 3 M NaSCN, 20 mM Tris, 1 M NaCl, 0.5 mM benzamidine, 2 mM EDTA was used.

A further suitable monoclonal antibody for purifying protein C has been described in US 5,336,610. This antibody binds to the activation peptide of protein C and thus is suitable to selectively purify the zymogen protein C from the activated form.

In detail: The prothrombin complex concentrate was dissolved in adsorption buffer, wherein approximately 10 g of the prothrombin complex concentrate were used for a 20 ml monoclonal antibody column. Subsequently,

the dissolved prothrombin complex concentrate was filtered, centrifuged for 15 min at 20,000 rpm, and sterile-filtered through a 0.8 μm filter. The sterile-filtered and dissolved prothrombin complex concentrate was applied to a column at a flow rate of 10 ml/h. Then the column was washed with a washing buffer so as to be free from protein, and finally the bound protein C was eluted with the elution buffer at a flow rate of 5 ml/h, and the fractions were collected. The eluted protein C was dialyzed against a buffer (0.2 M Tris, 0.15 M glycine, and 1 mM EDTA, pH 8.3). The protein C content was determind with respect to antigen by means of the method according to Laurell and with respect to activity after Protac activation.

The protein C eluate obtained was finished to a pharmaceutically applicable preparation in the following manner:

At first, the eluate was subjected to an ultrafiltration and a diafiltration step. For diafiltration, a buffer having a pH of 7.4 was used, which contained 150 mM NaCl and 15 mM trisodium citrate.2H$_2$O. The filtrate obtained was freeze-dried and virus inactivated by a one-hour vapor treatment at 80°C ± 5°C and 1375 ± 35 mbar.

The lyophilized, virus-inactivated material then was dissolved in a sterile isotonic NaCl solution, and possibly present antibodies or serum amyloid P,

respectively, were removed by means of ion exchange chromatography on Q-Sepharose. The purified solution was concentrated by a further ultrafiltration and diafiltration step. Thereafter, 10 g albumin, 150 mM NaCl and 15 mM trisodium citrate were added. The pH of the solution was 7.5. Murine immunoglobulin as well as the factors II, VII, IX and X could not be detected. Subsequently, the solution was sterile-filtered, filled into containers and lyophilized. The specific activity was 14 units of protein C/mg. As the activity assay, an amidolytic assay was used wherein protein C was activated by means of Protac (Pentapharm).

1.5 Purification of plasmatic protein S

Human protein S was prepared from factor IX concentrate (Prothrombin complex STIM-3, BAXTER AG, Vienna) by means of QAE-Sephadex and Blue Sepharose CL-6B chromatography (Pharmacia) in the following manner:

The lyophilized concentrate (100 g) was dissolved in 200 ml of sterile, ion-free water and dialyzed against a buffer consisting of 0.01 M 2-(N-morpholinoethane-sulfonic acid), pH 6.0; 0.18 M NaCl, 10 mM EDTA, 2 mM benzamidine-HCl and 0.02% NaN$_3$ (starting buffer). Then the dialyzed material was applied to a QAE-Sephadex column (8 x 19 cm) and equilibrated with the buffer mentioned. As washing solution, 1.5 l of buffer (starting buffer) were used.

Protein S was eluted at 110 ml/h with a linear NaCl

gradient consisting of 1.2 l of starting buffer and 1.2 l of a further buffer which differs from the first buffer by the addition of 0.5 M NaCl. The protein S fractions were examined for protein S by means of Fast Flow SDS PAGE (Pharmacia) and antigen determination (Laurell). Protein S-containing fractions were pooled and finally dialyzed against a buffer. This buffer contained 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM benzamidine-HCl and 0.2% NaN$_3$. After dialysis, the protein S pool was applied onto a Blue Sepharose column CL-6B (2.5 cm x 10.5 cm) and equilibrated with the starting buffer.

Washing was carried out with 500 ml of starting buffer at a flow rate of 15 ml/h. Thus, protein S could be eluted in the void volume, whereas prothrombin adsorbed to the column. Again, the protein S rich fractions were determined by means of SDS-PAGE Fast Flow System (Pharmacia) and according to Laurell (Scand. J. Clin. Invest. (Suppl.) 29 (1977) 21 (Suppl. 124)).

On a reduced SDS-PAGE, the thus prepared protein S had a morphology characteristic of protein S, namely two close bands (doublet) with a molecular weight of approximately 86,000 and 76,000, respectively. The protein concentration was determined spectrophoto-metrically by means of an extinction coefficient of 0.1 at 280 nm for human protein S and was confirmed by the

Method according to LOWRY (Lowry O. et al., Protein measurement with the Folin phenol reagent, J. Biol. Chem. 193 (1951) 265).

The thus prepared, pre-purified protein S was used for preparing sheep antiserum against protein S by carrying out four immunizing injections. 100 μg of protein S had been applied subcutaneously with Freund's adjuvant in the first two injections and incomplete adjuvant being employed in the following boosters. After further boosters, the antiserum was tested by means of double immunodiffusion and showed a precipitation with purified protein S and with normal plasma.

The IgG fraction from 450 ml antiserum was obtained by alcohol precipitation and subsequent adsorption on Sephadex A 50 in TRIS-HCl buffer, pH 6.8. From 450 ml of antiserum, 1.14 g of anti-protein S-IgG were present in the supernatant. The IgG fraction was coupled to 450 ml of Sepharose CL-4B, with 5.7 mg protein/ml Sepharose being used. The coupling efficiency was 76%. The anti-protein S column was equilibrated with glycine-HCl, pH 3, and adsorption buffer, pH 7.5.

The adsorption buffer was comprised of 20 mM TRIS, 2 mM EDTA, 0.25 M NaCl, 2 mM benzamidine, 0.02% Tween® 20 and 0.02% NaN_3, pH 7.4. The washing buffer solution had the following contents: 20 mM TRIS, 2 mM EDTA, 1.0 M NaCl, 0.5 mM benzamidine, 0.01% Tween® 20;

0.02% NaN$_3$, pH 7.4.

The elution buffer had a composition like the washing buffer solution, except that 0.05% Tween® 20 and, additionally, 243.3 g of NaSCN, pH 7.4 (a 3 M rhodanid solution) were used.

The dialysis buffer solution contained 20 mM Tris, 0.15 mM glycine, 1 mM EDTA, 2 mM benzamidine, pH 8.3.

For further purification of the protein S fraction prepared from prothrombin complex concentrate, 100 g of the fraction were dissolved in 1 l of adsorption buffer and dialyzed over night against an adsorption buffer solution. After application of the sample onto the column, the column was washed with washing buffer, approximately 5 l, to be free from protein, subsequently elution was carried out with 3 M NaSCN in the elution buffer solution. The eluate was dialyzed immediately until SCN was below the detection limit; the eluate had a concentration of 500 µg/ml protein S. It was free from C4-binding protein.

Monoclonal anti-protein S antibodies were prepared as follows:

BALB/C mice were immunized at two-week intervals by intraperitoneal injection of 100 µg of the protein S produced. After six weeks, once again 50 µg of the human protein S were injected, and 3 days afterwards fusion was carried out. The myeloma cell line (P3-X-63-AG8-653, 1.5 x 10^7 cells) was mixed with 1.7 x 10^8

- 32 -

spleen cells from a mouse, and fusion was effected according to a modified Köhler & Milstein method by using PEG 1500 (Köhler G., Milstein C., Nature 256 (1975), 495-497).

Positive clones, tested by means of an ELISA, were subcloned twice. Ascites production was effected by injection of 5×10^6 hybridoma cells per BALB/C mouse two weeks after Pristan treatment.

The immunoglobulin was purified from ascites by ammonium sulfate precipitation and subsequent chromatography by means of QAE-Sephadex and, thereafter, chromatography on Sephadex G200.

The IgG fraction obtained from ascites and pre-purified on protein A-Sepharose was coupled to Sepharose CL-4B. The affinity chromatographic purification of protein S which had been recovered from the prothrombin complex concentrate was effected under the conditions described for polyclonal protein S antibodies. The concentration of the eluate on protein S was 600 μg/ml. It was free from C4-binding protein.

The protein S preparations highly purified according to the method of polyclonal or monoclonal affinity chromatography were subjected to an SDS-PAGE (gradient gel 8 to 12%), and were designated as more than 95% pure according to Coomassie staining (Laemmli UK: Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970),

680).

Subsequently, the eluates were subjected to an ultrafiltration and a diafiltration step. For diafiltration, a buffer having a pH of 7.4 was used which contained 150 mM NaCl and 15 mM trisodium citrate.$2H_2O$. The filtrates obtained were freeze-dried and virus-inactivated by a one-hour vapor treatment at 80°C ± 5°C and 1375 ± 35 mbar (to remove possibly present viral contaminations of polyclonal or monoclonal antibody).

Then the lyophilized virus-inactivated material was dissolved in a sterile isotonic NaCl solution and possibly present antibodies and serum amyloid P, respectively, were removed by ion exchange chromatography on Q-Sepharose. The purified solution was concentrated by a further ultrafiltration and diafiltration step. Thereafter, 10 g of albumin, 150 mM NaCl and 15 mM trisodium citrate were added per liter to the solution obtained. The pH of the solution was 7.5. It contained 3000 µg/ml protein S. This content of protein S corresponds to a 500-fold enrichment as compared to plasma. Murine immunoglobulin as well as the factors II, VII, IX and X could not be detected. Subsequently, the solution was sterile-filtered, filled into containers and lyophilized.

E x a m p l e 2 : Pharmaceutical preparation of highly purified vitamin K-dependent proteins:

- 34 -

The highly purified single factors prothrombin,
factor VII, factor IX, factor X, protein C and protein
S were combined such that a solution containing
25,000 units/l of each of the single factors was formed
in aqueous solution of 4 g Na_3 citrate.$2H_2O$ and 8 g
NaCl/l. The solution was sterile-filtered via a nylon
filter having a retention rate of 0.2 μm and
subsequently filled at 20 ml each in sterilized glass
bottles having a filling volume of 50 ml. It was
freeze-dried under sterile conditions, whereafter the
bottles were closed under sterile conditions.

E x a m p l e 3 : Pharmaceutical preparation of
the prothrombin complex:

As described in the previous example, a highly
purified prothrombin complex comprising the factors
prothrombin, factor VII, factor IX and factor X, yet
without protein C and protein S, was prepared and
filled into containers under sterile conditions.
According to international recommendations, the
solution was admixed with 10,000 international units of
heparin/l before filling into containers.

E x a m p l e 4 : Pharmaceutical preparation of
vitamin K-dependent proteins for continuous
substitution and maintaining of constant plasma levels:

After substitution with a vitamin K-dependent
protein concentrate in which plasma levels as constant
as possible of the factors of the prothrombin complex

- 35 -

are to be attained, due to the different half-lives of the individual factors it is necessary to carry out the substitution rates of these proteins in a non-equivalent ratio. Accordingly, a pharmaceutical preparation as described above was produced which contained prothrombin, factor VII, factor IX, factor X, protein C and protein S at a ratio of 1:30:3:1.5:6:6.

E x a m p l e 5 : Pharmaceutical preparation of a partial prothrombin complex:

On account of the different *in vivo* half-lives of the factors of the prothrombin complex, in particular because of the long half-life of prothrombin of 2-5 days as compared to the short half-life of factor VII of 4-7 h, and of the factor IX of less than 24 h, in the continuous substitution with prothrombin complex preparations it happens that when adjusting a normal plasma concentration of 1 unit of factor VII/ml, already after 24 h the plasma concentration has decreased again to below the concentration of at least 20% required for a functioning haemostasis, whereas, e.g., the prothrombin plasma level still is approximately normal, i.e. is at 1 unit/ml. A repeated substitution with a prothrombin complex concentrate of the same composition as in the primary substitution will have the result that the prothrombin plasma concentration in the patient will be increased to above the normal range so as to attain once more equivalent

factor VII or factor IX plasma concentrations. Raising the plasma prothrombin concentration to above the standard range, however, means an increased risk of thrombosis for the patient treated, since Poort et al. (Blood 88:3698-3703, 1996) have found that a plasma level of prothrombin raised by as little as 30% involves a significantly increased risk of venous thromboses. This problem can be avoided by using combined prothrombin complex preparations for secondary substitutions.

Such a preparation is produces as follows: As described in the previous examples, a prothrombin complex concentrate comprising factor VII, IX and X at a ratio of 30:3:1.5, yet without prothrombin, is prepared and provided as a pharmaceutical formulation. The containers of this preparation intended for administration each contained 500 international units of the coagulation factors VII, IX and X in a dissolution volume of 10 ml after reconstitution of the lyophilized powder.

E x a m p l e 6 : Quality check of the prothrombin complex preparations:

The individual coagulation factors in the final containers of the prothrombin complex preparations were assayed for their content of prothrombin, factor VII, factor IX, factor X, protein C and protein S by means of 1-step coagulation tests according to standard

methods, by using deficient plasmas and coagulation reagents from BAXTER, Vienna.

Details to the methods used can be taken from the paper by Müller, H.G. and Bonik K. (Krankenhauspharmazie 13:528-531, 1992).

To determine the content of activated prothrombin complex factors, the chromogenic substrates S-2238, S-2222, S-2444, S-2366 and S-2251 (Chromogenix) were used. The determinations were carried out by using the buffer conditions indicated by the producer. With the chromogenic substrates, in each case the activated factors thrombin, factor VIIa, factor IXa, factor Xa, activated protein C which developed proteolytic activities against the peptide substrates used, could be determined. After evaluation of the photometric analysis of the turnover of the chromogenic substrate it could be determined that for all the substrates the content in the previously described preparation comprising vitamin K dependent proteins or selected factors of the prothrombin complex was less than 10 mU/ml in each case.

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprising" is used in the sense of

- 38 -

"including", i.e. the features specified may be associated with further features in various embodiments of the invention.

It is to be understood that a reference herein to a prior art document does not constitute an admission that the document forms part of the common general knowledge in the art in Australia or in any other country.

- 38A -

C l a i m s :

1.   A pharmaceutical, separated prothrombin complex
preparation, characterized in that it comprises at
least 2 chromatographically purified vitamin K-
dependent single factors as the active substances.

2.   A preparation according to claim 1, characterized
in that the single factors are selected from the group
of factor II, factor VII, factor IX, factor X, protein
C, protein S and protein Z.

3.   A preparation according to claim 1 or 2,
characterized in that it comprises at least the factors
II, VII, IX and X.

4.   A preparation according to any one of claims 1 to
3, characterized in that it comprises the single
factors protein C and protein S.

5.   A preparation according to any one of claims 1 to
4, characterized in that at least one of the single
factors is a recombinant factor, a transgenically
prepared factor, a derivative, in particular a peptide,
and/or a functional fragment.

6.   A preparation according to any one of claims 1 to

5, characterized in that it comprises the single
factors contained at a ratio which substantially
corresponds to the ratio of these factors in blood.

7. A preparation according to any one of claims 1 to
6, characterized in that it comprises the single
factors factor II, factor VII, factor IX and factor X
at relative ratios, based on international units, of
(0.5 to 2) : (0.5 to 2) : (0.5 to 2) : (0.5 to 2).

8. A preparation according to any one of claims 1 to
7, characterized in that it comprises the individual
factors factor II, factor VII, factor IX, factor X,
protein C and protein S at relative ratios, based on
international units, of (0.5 to 2) : (0.5 to 2) : (0.5
to 2) : (0.5 to 2) : (0.5 to 2) : (0.5 to 2).

9. A preparation according to any one of claims 1 to
5, characterized in that it comprises the single
factors contained at ratios which correspond to the
relative half-lives of the single factors.

10. A preparation acording to claim 9, characterized
in that it comprises the single factors factor II,
factor VII, factor IX and factor X at relative ratios,
based on international units, of (0.5 to 2) : (5 to 35)
: (0.5 to 7) : (0.5 to 5).

11. A preparation according to any one of claims 9 or 10, characterized in that it comprises the single factors factor II, factor VII, factor IX, factor X, protein C and protein S at relative ratios, based on international units, of (0.5 to 2) : (5 to 35) : (0.5 to 7) : (0.5 to 5) : (1 to 15) : (1 to 15).

12. A preparation according to claim 1, 2, 4, 5, 6 or 9, characterized in that it comprises factor VII and factor II at a ratio of greater than 10 : 1.

13. A preparation according to any one of claims 1 to 12, characterized in that the single factors in the preparation do not form a complex.

14. A preparation according to any one of claims 1 to 13, characterized in that it comprises a partial prothrombin complex.

15. A preparation according to any one of claims 1 to 14, characterized in that it does not contain an activated coagulation factor selected from IIa, IXa, Xa and, optionally, VIIa.

16. A preparation according to any one of claims 1 to 15, characterized in that it contains less than 0.1 U

of factor VIII:C or factor VIII:Ag/mg of protein.

17.   A preparation acording to any one of claims 1 to
16, characterized in that it contains less than 0.1 U
of factor IIa/U of prothrombin.

18.   A preparation according to any one of claims 1 to
17, characterized in that it contains less than 0.1 U
of factor Xa/U of factor X.

19.   A preparation according to any one of claims 1 to
18, characterized in that it is present in lyophilized
form.

20.   A preparation according to any one of claims 1 to
19, characterized in that it comprises magnesium ions.

21.   A preparation according to any one of claims 1 to
20, characterized in that it does not contain free
calcium ions.

22.   A preparation according to claim 21,
characterized in that it comprises a chelating agent
for complexing free calcium ions.

23.   A preparation according to any one of claims 1 to
22, characterized in that it further comprises

antithrombin III in stabilizing amounts, optionally together with heparin.

24.   A preparation according to any one of claims 1 to 23, characterized in that it is free from infectious viruses on account of a virus inactivation or virus depletion treatment.

25.   A preparation according to claim 24, characterized in that its freedom from infectious viruses is ensured by two independent virus inactivation or virus depletion methods.

26.   A preparation according to any one of claims 1 to 25, characterized in that it further comprises pharmaceutically acceptable buffer substances or stabilizers.

27.   A preparation according to any one of claims 1 to 26, characterized in that it comprises highly purified vitamin K-dependent single factors which are virus-inactivated.

28.   A preparation according to any one of claims 1 to 27, characterized in that it comprises the single factor X in its α-form and/or in its β-form.

29.   A diagnostical preparation, comprising a
preparation according to any one of claims 1 to 28.

30.   The use of a preparation according to any one of
claims 1 to 28 for producing a preparation for treating
acquired or inherited blood coagulation disorders.

31.   The use of a preparation according to any one of
claims 1 to 28 for producing a preparation for treating
severe hemorrhages.

32.   The use of a preparation according to any one of
claims 1 to 28 for producing a preparation for the
prophylaxis of hemorrhages, in particular in case of
inherited blood coagulation disorders.

33.   The use of a preparation according to any one of
claims 1 to 28 for producing a preparation for
substitution therapy.

34.   The use of a preparation according to any one of
claims 1 to 28 for producing a preparation for treating
hemophilia B.

35.   A method of treating acquired or inherited blood
coagulation disorders in a patient, comprising
administering to the patient a preparation according to
any one of claims 1 to 28.

5

36.   A method of treating a severe haemorrhage in a
patient comprising administering to the patient a
preparation according to any one of claims 1 to 28.

10   37.   A method for the prophylaxis of haemorrhage in a
patient comprising administering to the patient a
preparation according to any one of claims 1 to 28.

38.   A method of substitution therapy of a patient
15   comprising administering to the patient a preparation
according to any one of claims 1 to 28.

39.   A method of treating haemophilia B in a patient
comprising administering to the patient a preparation
20   according to any one of claims 1 to 28.

40.   A preparation according to claim 1 substantially as
described herein with reference to the accompanying
Examples.

25

Dated this 16th day of November 2001
BAXTER AKTIENGESELLSCHAFT
By their Patent Attorneys
GRIFFITH HACK